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Studies of *Neurospora crassa* folylpolyglutamate synthetase: Elucidation of the *met-6*, *mac* and *met-S2706* mutations.

by

Ian Joseph Atkinson ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

in

Plant Biochemistry

Department of Botany

Edmonton, Alberta

Spring, 1995

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Studies of Neurospora crassa folylpolyglutamate synthetase: Elucidation of the met-6, mac and met-s2706 mutations, submitted by Ian Joseph Atkinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Biochemistry.

Dr. E.A. Cossins

Dr. F. E. Nargang

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Dr. B. Shane

Dr. A. Horak

March 9, 1995

Dedication

This thesis I dedicate to my father,

may he rest in peace.

Abstract

The methionine auxotrophy of Neurospora crassa met-6, mac and met-s2706 mutants is related to lesions affecting folylpolyglutamate synthetase (FPGS) activity, however, it is not clear whether these mutations occur in different genes or in different functional domains of the same gene. To address this question, copies of the N. crassa met-6$^+$ gene were introduced into mutant spheroplasts by transformation with cosmid and plasmid vectors. Transformants to prototrophy were produced in all mutant strains. The 3.5 kb EcoRI-BamHI fragment from wild-type strain 74-OR23-1A that rescues all three mutants was sequenced, and one open reading frame (ORF) was identified. This ORF contains several AUG codons, and translation beginning from either of the first two would produce a protein of appropriate size that is homologous to four other FPGS proteins. The position of the transcription stop site and of an intron were identified by sequencing cDNA clones which were truncated at the 5' end. DNA sequence analysis of the met-6 mutant allele revealed a T to C transition which resulted in the replacement of the wild type serine with a proline. The serine in the wild-type protein is present in a highly conserved region of the protein and might be at or near the active site of the protein. Northern analysis of met-6$^+$ transcripts revealed a single 2.0 kb product, significant levels of which were present only early in germination.

The ability of mutant and transformant strains to synthesize polyglutamates was assessed by HPLC analysis of folate cleavage products. Analysis of endogenous polyglutamates or analysis of incorporated [^{14}C]p-ABA into mycelial polyglutamates showed that transformant and wild type cultures synthesized long chain length folates, the majority of these being hexaglutamyl derivatives. In contrast, folates of met-6 and mac were mainly short chain length derivatives. Met-s2706 mutant pools were hexaglutamyl folate, but total folate levels of [^{14}C]p-ABA incorporation were observed to be $\leq$ 10% of wild type levels. Polyglutamate synthesis was also studied in vitro by

partial purification and characterization of mycelial FPGS protein. Mycelial extracts of the wild-type and transformant cultures utilized 5,10-methylenetetrahydrofolate monoglutamate and its diglutamate as substrates in the synthetase reaction. In contrast, extracts of met-6 and mac/met-s2706 mycelia utilized only one of these folate substrates respectively. Gel filtration of FPGS protein indicated apparent molecular weight values of 66 kD in all strains. Wild type FPGS protein was purified over 56,000-fold and possessed the ability to conjugate mono- and diglutamate folate substrates and did not show any separation of the activities upon purification. The primary band was of apparent molecular weight 60 kD with only 3 silver staining bands remaining on SDS-PAGE. It is suggested that polyglutamate synthesis in Neurospora is probably mediated, as in other eukaryotic species, by a single gene encoding a single FPGS protein. Thus, the deficiencies in the mutants are likely due to mutations in the same enzyme, not different enzymes. This conclusion is supported by the observation that transformation with 2.2 kb PCR fragments of wild-type or complementary mutant DNA can transform all mutants to prototrophy. Therefore the mutants should be renamed as three mutant alleles of a single gene.

Acknowledgments

I would like to take this opportunity to thank my supervisor, Dr. Edwin A. Cossins, for his many words of encouragement, guidance and support during the completion of the research and during the preparation of the thesis. His advice was honest, his assistance was given freely, and his help was much needed and appreciated.

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I also thank Dr. Tom Schmidhauser for his generous gift of the cosmids needed to start the project in the first place.

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List of Abbreviations

ADP	adenosine diphosphate
AICAR	5-phosphoribosyl-5-amino-4-imidazole
Amp^r	ampicillin resistance
ATP	adenosine triphosphate
bp; kb	base pairs; kilobase pairs
bromophenol blue	3', 3", 5', 5", -tetrabromophenol-sulphonphthalein
BSA	bovine serum albumin
C_1-metabolism	one-carbon metabolism
C_1-THF Synthase	multifunctional C_1 tetrahydrofolate synthase
cDNA	complementary DNA
CHO	chinese hamster ovary
Ci; µCi; mCi	Curie; microCurie; milliCurie
Da; kDa	Daltons; kiloDaltons
dATP; ddATP	2'-deoxyadenosine 5'-triphosphate; 2', 3', -dideoxyadenosine 5'- triphosphate
dCTP; ddCTP	2'-deoxycytidine 5'-triphosphate; 2', 3', -dideoxyacytidine 5'- triphosphate
dGTP; ddGTP	2'-deoxyguanosine 5'-triphosphate; 2', 3', -dideoxyguanosine 5'- triphosphate
dITP; ddITP	2'-deoxyinosine 5'-triphosphate; 2', 3', -dideoxyinosine 5'- triphosphate
dTTP; ddTTP	2'-deoxythymidine 5'-triphosphate; 2', 3', -dideoxythymidine 5'- triphosphate
DEAE	diethylaminoethyl

DEPC	diethylpyrocarbonate
dH_2O; ddH_20	distilled water; double distilled water
DHFR	dihydrofolate reductase
DHFS	dihydrofolate synthetase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FGSC	Fungal Genetic Stock Center
fMet-tRNA	formylmethionyl-transfer RNA
GAR	5-phosphoribosylglycinamide
GGH	gamma glutamyl hydrolase
HPLC	high pressure liquid chromatography
hr; min	hour; minute
IPTG	isopropyl-ß-D-thiogalactoside
MOPS	3-(N-morpholino)propanesulphonic acid
MTX	methotrexate
NAD; NADP	nicotinamide adenine dinucleotide; nicotinamide adenine dinucleotide phosphate
N. crassa	*Neurospora crassa*
NTPs; dNTPs	nucleoside triphosphates; 2'-deoxynucleoside triphosphates
p-ABA	p-aminobenzoate
p-$ABAGlu_n$	p-aminobenzoyl polyglutamate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PEG	polyethylene glycol
Pi	inorganic orthophosphate
PMSF	phenylmethylsulphonylfluoride
H_2Pte	dihydropteroic acid
PteGlu	pteroylglutamic acid
$H_2PteGlu$	dihydropteroylglutamate; dihydrofolate
$H_4PteGlu$	tetrahydropteroylglutamate; tetrahydrofolate
$H_4PteGlu_n$	tetrahydropteroylpolyglutamate, n indicates the number of L-glutamate moieties
10-HCO-$H_4PteGlu_n$	N^{10}-formyl-tetrahydrofolate polyglutamate
5,10-CH_2-$H_4PteGlu_n$	N^5,N^{10}-methelene-tetrahydrofolate polyglutamate
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
rpm	revolutions per minute
SAX	strong anion exchange
SHMT	serine hydroxymethyltransferase
SDS	sodium dodecyl sulphate
Tet^r	tetracycline resistance
THFA	tetrahydrofolic acid
Tris	tris (hydroxymethyl) aminomethane
Ts	Thymidylate synthase
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

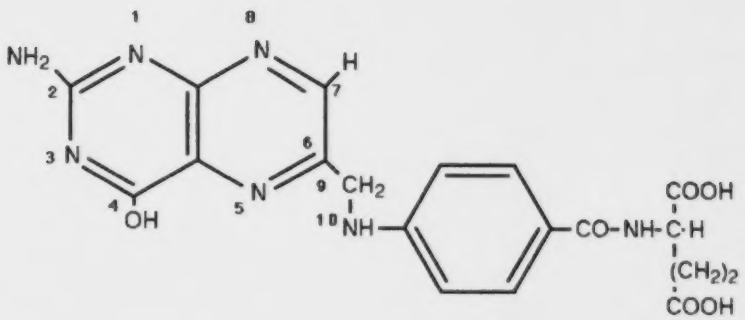
1. Introduction

Nutritional studies using various extracts (Table 1) led to the discovery and isolation of a closely related group of compounds containing N-[4-{[(2-amino-4-hydroxy-6-pteridinyl)methyl]amino}benzoyl]glutamic acid (Angiers et al., 1946). These compounds came to be more commonly known as folates, a term originating from Mitchell and his group (Mitchell et al., 1941, 1944; Frieden et al., 1944; Mitchell and Williams, 1944; Mitchell, 1944) to describe the Streptococcus faecalis R. nutritional factor, "folic acid", isolated from spinach leaves (Figure 1). Folate compounds were identified as essential nutrients that could serve as growth factors for chicks and lactic acid bacteria, and as the treatment of macrocytic anemias and tropical celiac sprue (Table 1).

Folates are an ubiquitous class of compounds which serve as carriers of individual carbon units in a variety of reactions involved in amino acid and nucleotide metabolism, collectively known as one-carbon metabolism (Blakley, 1969; MacKenzie, 1984; Cossins, 1984; Shane, 1989). Folates function directly or indirectly as coenzymes in the construction of purines, pyrimidines, methionine, and formylmethionyl tRNA (Blakley, 1969). Research into folate metabolism and biochemistry has increased dramatically in the last 50 years, and has attracted considerable attention.

Recent studies have provided insights into the significance of folylpolyglutamates in folate biochemistry. Folylpolyglutamates act as intracellular substrates and/or inhibitors of the enzymes of C_1 metabolism (Kisliuk, 1981; McGuire and Bertino, 1981; McGuire and Coward, 1984) while the corresponding monoglutamate derivatives are ineffective or poor substrates/inhibitors. Folylpolyglutamates are also required for normal cellular retention of folates (Schirch and Strong, 1989) whereas the monoglutamyl folate is the form transported across membranes. The reader's attention is directed to a selection of reviews on these subjects (Cossins, 1980, 1987; Kisliuk, 1981; MacKenzie, 1984; McGuire and Coward, 1984; Schirch and Strong, 1989; Shane, 1989) for further information. These topics will be reviewed in more detail in subsequent sections of the Introduction.

FOLIC ACID (PTEROLYGLUTAMATE)



TETRAHYDROFOLATE POLYGLUTAMATE

$H_4PteGlu_n$

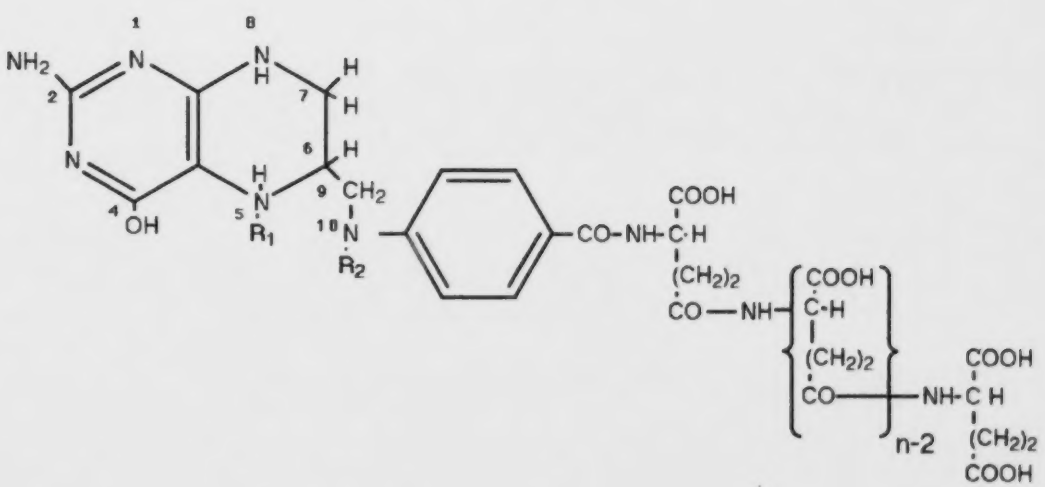


Figure 1. Structure of folic acid and tetrahydrofolate polyglutamate

Table 1. Observations Leading to Isolation and Synthesis of Folic Acid and Related Compounds

Reprinted with slight modifications from Spies, 1947.

Name	Source	Biological Activity	Chemical Nature
Norite eluate factor	Liver and Yeast	Growth factor for *L. casei* and *S. lactis*	Basic; related to purines
Norite eluate factor	Solubilized Liver	Growth factor for *L. casei* and *S. lactis*	Acidic; not a nucleotide
Folic acid	Spinach	Growth factor for *L. casei* and *S. lactis*	Not a nucleotide
S. lactis R. factor	Unstated	Active for *S. lactis* ; inactive for *L. casei*	
L. casei factor	Liver and Yeast	Liver factor active for both organisms; yeast factor half active for *S. lactis.*	Crystalline; Methyl esters of active principle
Folic acid	Spinach	Same as folic acid above	Xanthopterin-like structure
Marmite	Yeast	Cures dietary anemias in monkeys	
Vitamin M	Liver and Yeast	Cures nutritional cytopenias in monkeys	
Xanthopterin	Synthetic	Partially cures cytopenia in monkeys; cures trout anemias	
Vitamin B_c	Liver, Yeast Digest	Growth factor for chick, *L. casei* and *S. lactis.* Cures chick anemia	Crystalline; Acidic; similar to flavins, aloxozines and pterins
Factors R and S	Yeast	Required in chick nutrition	
Vitamin B_c conjugate	Yeast	Active for chick; 2-5% activity for *L. casei* and *S. lactis*	As above; molecule 2.8 larger
L casei factor	Unspecified	Active for *S. lactis* ; inactive for *L. casei* unlike folic acid	Crystalline; Absorption spectrum
L. casei factor	Synthetic	Active for chick, rat, monkey, bacteria	Crystalline

The Introduction to this thesis is divided into several sections. The first is a review of one-carbon metabolism. Focus is placed on folate-dependent reactions that donate one-carbon units that lead to purine, thymidylate, methionine and formylmethionyl-tRNA production. The second section examines the biological occurrence of conjugated folylpolyglutamates. Their role in cells and their function in the regulation of homeostasis are briefly examined. Third, the nature of the activities by which folylpolyglutamates are synthesized and degraded, and some of the general properties of these activities, are investigated. Finally, Neurospora folylpolyglutamate synthesis is discussed, with an emphasis on areas relevant to research data presented in this thesis.

1.1. The Biochemistry of Folate and One-Carbon Metabolism

1.1.1. The Nature of the Folate Molecule

As a class of compounds, folates consist of three major constituents (Figure 1):

1. a pteridine ring linked via a methylene group at the C-6 position to
2. a 4-aminobenzoate moiety and
3. a glutamate residue .

Studies of living organisms have shown that folates tend not to exist as fully oxidized folic acid, but are present predominantly as reduced forms (Blakley, 1969). Coenzymatically active folic acid is reduced at positions 7 and 8 of the pteridine ring to give a dihydrofolate form, or reduced even further to a more active tetrahydrofolate form at positions 5, 6, 7 and 8 of the pteridine ring (Figure 1). One-carbon metabolism requires the acquisition and substitution by oxidation/reduction of carbon units at the N^5 atom to give 5-formyl, 5-formimino and 5-methyl derivatives (Fig. 1; R_1 at N^5), at the N^{10} atom to give 10-formyl derivatives (Fig. 1; R_2 at N^{10}) or at the N^5 and N^{10} positions to form derivatives that occur as 5,10-methylene and 5,10-methenyl bridge structures (Fig. 1; a carbon bridge between R_1 and R_2). The folate molecule does not remain enzyme bound, but acts rather as a cosubstrate (Shane, 1989). Consequently the ability of folate to act as a donor of one

carbon units depends on the regeneration of its one-carbon moiety by other enzymes in the various cycles. Folates in solution are sensitive to oxygen, light, and extremes of pH (Mullins and Duch, 1992).

Accumulated evidence indicates that endogenous tissue folates exist primarily as pteroylpolyglutamates (Figure 1; Baugh et al., 1974; Brown et al., 1974; McGuire and Coward, 1984; Cossins, 1980, 1984; Shane, 1989), the glutamate chain joined via γ-glutamyl peptide bonds containing on average 5 to 8 glutamate residues (Cossins, 1984). These structures are also known as folylpolyglutamates, polyglutamyl folates, or simply as polyglutamates. This topic will be explored in detail in subsequent sections.

1.1.2. Synthesis of the Folate Molecule

Evidence for a pathway for folate synthesis de novo was provided by early nutritional studies using charcoal-treated cell-free extracts and labeled precursors (see review by Stokstad, 1990). These studies suggested a mechanism of folic acid biosynthesis as follows:

GTP---------> 6-hydroxymethyldihydropteridine ---> 6-pyrophosphorylmethyl (1a)
+ MgATP dihydropteridine intermediate

6-pyrophosphorylmethyldihydropteridine ----------> dihydropteroic acid (1b)
intermediate + p-ABA

dihydropteroic acid + L-glutamate ----------> dihydrofolic acid (2)

dihydrofolic acid + $NADPH+H^+$ ---------> tetrahydrofolic acid + $NADP^+$ (3)

The synthesis involves the conversion of GTP into a pterin precursor (7,8-dihydro-6-hydroxymethylpteridine). This compound is then converted to a pyrophosphate intermediate by a reaction involving ATP and Mg^{2+}. The pyrophosphate intermediate

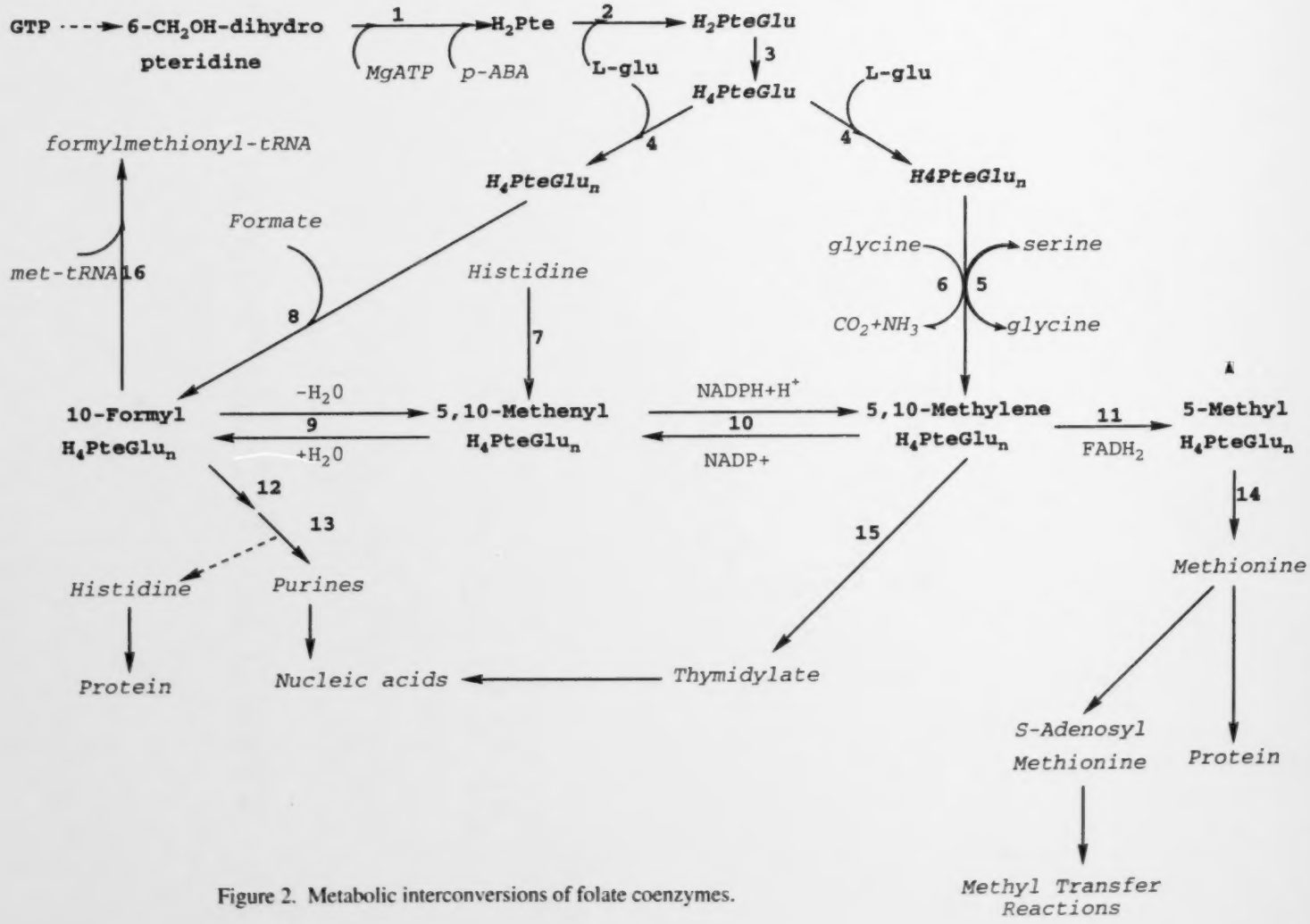


Figure 2. Metabolic interconversions of folate coenzymes.

condenses with p-ABA to yield dihydropteroate (Shiota et al., 1964; Cossins, 1980). These first steps (Figure 2, reactions 1a & 1b) are catalyzed by two enzymes, pteridine phosphokinase (Table 2, reaction 1a; Richey and Brown, 1971) and dihydropteroate synthetase (DHPS; Table 2, reaction 1b; Shiota et al., 1969). Sulfanilamides, which are analogues of p-aminobenzoic acid, inhibit incorporation of p-aminobenzoic acid into folic acid via the DHPS reaction, thus inducing folate deficiency in organisms that require de novo folate synthesis (Brown, 1962).

Dihydrofolate synthetase (DHFS; Figure 2, Table 2, reaction 2) catalyzes the generation of dihydrofolate from dihydropteroate. Bacteria, plants and fungi were shown to have DHFS activity and the capacity of de novo folate synthesis, whereas Ikeda and Iwai (1975) showed that mammalian tissues lacked DHFS activity. This inability to generate dihydrofolate accounts for the dietary requirement of mammals for folates as vitamins.

Dihydrofolate is converted to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR; Figure 2, Table 2, reaction 3). This enzyme has been studied in great detail with mammals, bacteria, and plants. The two major roles of this enzyme are de novo tetrahydrofolate synthesis and reduction of dihydrofolate after thymidylate synthesis (see section 1.1.3.3.). In both cases DHFR is involved in the reduction of dihydrofolate to tetrahydrofolate. This enzyme will be reviewed in greater detail under thymidylate synthesis (section 1.1.3.3).

1.1.3. An Overview of One-Carbon Metabolism

An overall view of the various metabolic cycles of one-carbon metabolism in prokaryotes and eukaryotes is shown in Figure 2, and the enzymes involved are shown in Table 2. These reactions can be divided into four groups: the generation of C_1 units, the interconversion of C_1 units, the incorporation of C_1 units into new compounds, and the disposal of C_1 units. A brief review of these four groups of reactions is presented.

Table 2. The Major Enzymes of Folate-Mediated One-Carbon Metabolism.

1a.	Trivial name(s) -	2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine pyrophosphokinase
	Systematic name -	2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine 6'-pyrophosphotransferase; EC# 2.7.6.3
	Reaction -	ATP + 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine = AMP + 2-amino-7,8-dihydro-4-hydroxy-6-
1b.	Trivial name(s) -	Dihydropteroate synthase; dihydropteroate pyrophosphorylase
	Systematic name -	2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate: 4-aminobenzoate 2-amino-4-hydroxy-dihydropteridine-
		6- methenyl transferase; EC# 2.5.1.5
	Reaction -	2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate + 4-aminobenzoate = pyrophosphate + dihydropteroate
2.	Trivial name(s) -	Dihydrofolate synthetase; dihydrofolate synthase
	Systematic name -	dihydropteroate: L-glutamate ligase (ADP forming); EC# 6.3.2.12
	Reaction -	ATP + dihydropteroate = ADP + Pi + dihydrofolate
3.	Trivial name(s) -	Dihydrofolate reductase; tetrahydrofolate dehydrogenase
	Systematic name -	5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase; EC# 1.5.1.3
	Reaction -	7,8-dihydrofolate + NADPH = 5,6,7,8-tetrahydrofolate + $NADP^+$
4.	Trivial name(s) -	Folylpolyglutamate synthetase; tetrahydrofolylpolyglutamate synthase
	Systematic name -	tetrahydrofolate: L-glutamate γ-ligase (ADP forming); EC# 6.3.2.17
	Reaction -	ATP + $H_4PteGlu_n$ + L-glutamate = ADP + Pi + $H_4PteGlu_{n+1}$

Table 2 (Con't)

5.	Trivial name(s) -	Serine hydroxymethyltransferase; glycine hydroxymethyltransferase; serine hydroxymethylase; serine aldolase; threonine aldolase
	Systematic name -	5,10-methylene: glycine hydroxymethyltransferase; EC# 2.1.2.1
	Reaction -	5,10-methylenetetrahydrofolate + glycine + H_2O = tetrahydrofolate + serine
6a.	Trivial name(s) -	Glycine dehydrogenase (decarboxylating); P protein; glycine decarboxylase
	Systematic name -	glycine: lipoylprotein oxidoreductase (decarboxylating and acceptor-aminomethylating); EC# 1.4.4.2
	Reaction -	glycine + lipoylprotein = S-aminomethyldihydrolipoylprotein + CO_2
6b.	Trivial name(s) -	Aminomethyltransferase; T protein; tetrahydrofolate aminomethyltransferase
	Systematic name -	S-aminomethyldihydrolipoylprotein: (6S)tetrahydrofolate aminomethyltransferase (ammonia forming); EC# 2.1.2.10
	Reaction -	S-aminomethyldihydrolipoylprotein + (6S)tetrahydrofolate = dihydrolipoylprotein + (6R)5,10-methylenetetrahydrofolate + NH_3
7a.	Trivial name(s) -	Glutamate formimino transferase
	Systematic name -	5-formiminotetrahydrofolate: L-glutamate N-formiminotransferase; EC# 2.1.2.5
	Reaction -	5-formiminotetrahydrofolate + L-glutamate = tetrahydrofolate + N-formiminoglutamate
7b.	Trivial name(s) -	Formiminotransferase; glycine formimino transferase
	Systematic name -	5-formiminotetrahydrofolate: glycine N-formiminotransferase; EC# 2.1.2.4
	Reaction -	5-formiminotetrahydrofolate + glycine = tetrahydrofolate + N-formiminoglycine
7c.	Trivial name(s) -	5-formiminotetrahydrofolate cyclodeaminase
	Systematic name -	5-formiminotetrahydrofolate ammonia lyase (cyclizing); EC# 4.3.1.4
	Reaction -	5-formiminotetrahydrofolate = 5,10-methenyltetrahydrofolate + NH_3

Table 2 (Con't)

7d.	Trivial name(s) -	Glutamate formyl transferase
	Systematic name -	N-formyl-L-glutamate: tetrahydrofolate 5-formyltransferase; EC# 2.1.2.6
	Reaction -	5-formyltetrahydrofolate + L-glutamate = tetrahydrofolate + N-formylglutamate
7e.	Trivial name(s) -	formyltetrahydrofolate cyclodehydrase
	Systematic name -	5-formyltetrahydrofolate cyclo-ligase, ADP forming; EC# 6.3.3.a
	Reaction -	5-formyl-H_4PteGlu + MgATP -----> [5,10-methenyl-H_4PteGlu]$^+$ + MgADP + Pi
8a.	Trivial name(s) -	formyltetrahydrofolate synthetase; formate-tetrahydrofolate ligase
	Systematic name -	formate: tetrahydrofolate ligase (ADP forming); EC# 6.3.4.3
	Reaction -	ATP + formate + tetrahydrofolate = ADP + Pi + 10-formyltetrahydrofolate
8b.	Trivial name(s) -	Formate-dihydrofolate ligase
	Systematic name -	formate: dihydrofolate ligase (ADP forming); EC# 6.3.4.17
	Reaction -	ATP + formate + dihydrofolate = ADP + Pi + 10-formyldihydrofolate
9.	Trivial name(s) -	Methenyltetrahydrofolate cyclohydrolase
	Systematic name -	5,10-methylenetetrahydrofolate 5-hydrolase (decyclizing); EC# 3.5.4.9
	Reaction -	[5,10-methenyl-H_4PteGlu]$^+$ + H_2O => 10-formyl-H_4PteGlu$_n$ + H+
10.	Trivial name(s) -	5,10-methylenetetrahydrofolate dehydrogenase
	Systematic name -	5,10-methylenetetrahydrofolate: $NADP^+$ oxidoreductase; EC# 1.5.1.5
	Reaction -	5,10-methylene-H_4PteGlu + $NADP^+$ => [5,10-methenyl-H_4PteGlu]$^+$ + NADPH

Table 2 (Con't)

11. Trivial name(s) -	5,10-Methylene tetrahydrofolate reductase (NADPH)
Systematic name -	5-methyltetrahydrofolate: $NADP^+$ oxidoreductase; EC# 1.5.1.20 (see also 1.7.99.5)
Reaction -	5-methyltetrahydrofolate + $NADP^+$ = 5,10-methylenetetrahydrofolate + NADPH
12. Trivial name(s) -	Glycinamide ribotide (GAR) transformylase
Systematic name -	10-formyltetrahydrofolate: 5' phosphoribosylglycinamide N-formyltransferase; EC# 2.1.2.2
Reaction -	10-formyltetrahydrofolate + 5'-phosphoribosylglycinamide = tetrahydrofolate + 5'-phosphoribosyl-N-formylglycinamide
13. Trivial name(s) -	Phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase
Systematic name -	10-formyltetrahydrofolate: 5' phosphoribosylaminoimidazolecarboxamide N-formyltransferase; EC# 2.1.2.3
Reaction -	10-formyltetrahydrofolate + 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide = tetrahydrofolate + 5'-phosphoribosyl-5-formamido-4-imidazolecarboxamide
14a.Trivial name(s) -	Methionine synthase (B_{12} dependent); tetrahydrofolate methyltransferase
Systematic name -	5-methyltetrahydrofolate: homocysteine S-methyltransferase; EC# 2.1.1.13
Reaction -	5-methyltetrahydrofolate + L-homocysteine = tetrahydrofolate + L-methionine
14b.Trivial name(s) -	Methionine synthase (B_{12} independent); tetrahydropteroyltriglutamate methyltransferase
Systematic name -	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase; EC# 2.1.1.14
Reaction -	See Reaction 14a
15. Trivial name(s) -	Thymidylate synthase
Systematic name -	5,10-methylenetetrahydrofolate: dUMP C-methyltransferase; EC# 2.1.1.45
Reaction -	5,10-methylenetetrahydrofolate + dUMP = dihydrofolate + dTMP

Table 2 (Con't)

16. Trivial name(s) -	Methionyl-tRNA transformylase
Systematic name -	10-formyltetrahydrofolate: L-methionyl tRNA N-formyltransferase; EC# 2.1.2.9
Reaction -	10-formyltetrahydrofolate + L-methionyl tRNA + H2O = tetrahydrofolate + *N* -formylmethionyl tRNA
17a. Trivial name(s) -	Folate conjugase; pteroyl-poly-γ-glutamate hydrolase; lysosomal GGH
Systematic name -	γ-glutamate-x carboxypeptidase; EC# 3.4.19.9
Reaction	$PteGlu_n = PteGlu + L\text{-}glu_{n-1}$ or $PteGlu_n = PteGlu_{n-1} + L\text{-}glu$
17b. Trivial name(s) -	Microsomal GGH; jejunal brush border exopeptidase
Systematic name -	pteroylpoly-γ-glutamate carboxypeptidase; EC# 3.4.19.8
Reaction	$PteGlu_n = PteGlu_{n-1} + L\text{-}glu$

1.1.3.1 The Generation of One-Carbon Units.

The general requirement of rapidly dividing cells for one-carbon units implies that mechanisms exist to bring carbon into the folate pool. One-carbon units are generated from primarily four compounds: serine, glycine, histidine and formate (Figure 2 - Reactions 5, 6, 7 and 8). Of these compounds, serine and glycine are quantitatively the most important contributors (MacKenzie, 1984; Shane, 1989).

Serine-Glycine Interconversion

Serine and glycine generate 5,10-methylenetetrahydrofolate, catalyzed by the enzymes serine hydroxymethyltransferase (SHMT; Figure 2, Table 2, reaction 5), and the glycine cleavage system (Figure 2, Table 2, reaction 6). The 5,10-methylene-H_4PteGlu formed in these reactions is central to one-carbon metabolism, as the one-carbon moiety can be directly incorporated into thymidylate synthesis, or have an indirect role in the production of 5,10-methenyl- or 5-methyl-H_4PteGlu for use in other one-carbon cycles.

Serine hydroxymethyltransferase (previously termed serine hydroxymethylase), a pyridoxal phosphate containing enzyme with four identical subunits (Schirch, 1982), catalyzes the transfer of formaldehyde from the ß-carbon of serine to H_4PteGlu to generate 5,10-methylene-H_4PteGlu and glycine as follows (Figure 2, reaction 5):

$$\text{Serine} + H_4\text{PteGlu} <=> \text{Glycine} + \text{5,10-methylene-}H_4\text{PteGlu} + H_2O \qquad (5)$$

Serine hydroxymethyltransferase catalyzes a completely reversible reaction, and, as such, plays a role in the formation of both glycine and serine (Schirch, 1984). A number of recent studies have shown that the hydroxymethyltransferase exists as two immunologically distinct isozymes, one cytosolic and one mitochondrial (reviewed by Cossins, 1987). Investigations of SHMT mutant cell lines have drawn attention to the complexity of the interactions between the cytosolic and mitochondrial compartments that influence one carbon unit generation (Chasin et al., 1974; Taylor and Hanna, 1982;

Appling, 1991).

The glycine cleavage system catalyzes the oxidative decarboxylation of glycine, where the 2-carbon of glycine enters the folate pool, and one molecule of each 5,10-methylene-H_4PteGlu, CO_2, and NH_3 are formed as follows (Kikuchi, 1973):

$$\text{Glycine} + H_4\text{PteGlu} + NAD^+ \rightarrow 5,10\text{-}CH_2H_4\text{PteGlu} + NADH + CO_2 + NH_3 \quad (6)$$

It is doubtful whether the synthetic reaction to form glycine is of physiological significance (Okamura-Ikeda et al., 1987). The glycine cleavage system is a complex of four closely associated proteins, designated as the P-, H-, L-, and T- subunits that have been isolated from bacterial cells (Sagers and Gunsalus, 1961), mammalian mitochondria (Hiraga and Kikuchi, 1980; Schirch, 1984), and plant mitochondria (Neuberger et al., 1991). For a review on the reaction mechanism, the reader is directed to Douce and Neuburger (1990).

Coupling of the serine hydroxymethyltransferase with the glycine cleavage system appears to be of prime importance in photosynthetic organisms for mitochondrial serine synthesis during photorespiration (Somerville and Somerville, 1986; Kendall et al., 1983). The reactions catalyzed by this complex provide a mechanism for the mitochondrial synthesis of serine from glycine, and is the principal source of CO_2 loss in photorespiration (Key, 1980; Tolbert, 1980).

Formate Incorporation

One-carbon substituted folates may also be generated from formate (Figure 2). An important source of one-carbon units in plants is photorespiration in generating one-carbon units by way of glyoxylate and glycine. 10-formyl-H_4PteGlu can then be obtained by a direct formylation of H_4PteGlu, catalyzed by the enzyme formyltetrahydrofolate synthetase as follows:

$$\text{formate} + MgATP + H_4\text{PteGlu} \Rightarrow 10\text{-formyl-}H_4\text{PteGlu} + MgADP + Pi + H^+ \quad (8)$$

This enzyme is associated as a trifunctional complex with the methylenetetrahydrofolate

dehydrogenase and methenyltetrahydrofolate cyclohydrolase in mammalian tissues (Tan et al., 1977; Schirch, 1978) and in yeast (Appling and Rabinowitz, 1985a,b,c). This complex has been named the C_1-THF synthase (see also section 1.1.3.2). Recent plant studies in spinach leaves (Nour and Rabinowitz, 1992) and pea cotyledons (Kirk et al., 1994) have shown that these tissues contain a monofunctional formyltetrahydrofolate synthetase protein, similar to bacteria, yet spinach had greater amino acid homology to mammalian synthetase domains than to bacterial or yeast proteins (Nour and Rabinowitz, 1992).

Histidine Metabolism

The reader is directed to reviews by Shane (1989) and Blakley (1969) for detailed information on this topic, including information on formylglutamate, formiminoglycine and 5-formyltetrahydrofolate metabolism. The breakdown of histidine (Figure, 2, reaction 7) in certain bacteria and liver occurs via the compound formiminoglutamate (FIGLU), which in the presence of tetrahydrofolate produces glutamic acid and formiminotetrahydrofolate (Table 2, reaction 7a; Miller and Waelsh, 1954; Tabor and Rabinowitz, 1956). The enzyme glutamate formiminotransferase, located in the cytoplasm, catalyzes the transfer of a formimino group from FIGLU to $H_4PteGlu_n$ as follows:

formiminoglutamate+ H_4PteGlu => 5-formimino-H_4PteGlu + glutamate (7a)

Under conditions of folate or vitamin B_{12} deficiency, formiminoglutamate catabolism is impaired and FIGLU is excreted in elevated amounts in urine (Silverman et al., 1952), useful as a diagnostic indicator of clinical folate deficiency. The formimino moiety cannot be utilized as such in one-carbon metabolism and has to be converted to 5,10-methenyl-$H_4PteGlu_n$ in a formiminotetrahydrofolate cyclodeaminase catalyzed reaction (Table 2, reaction 7c):

5-formimino-H_4PteGlu+H^+ => 5,10-methenyl-$H_4PteGlu_n$ + NH_3 (7c)

In mammalian tissues, formiminotransferase and cyclodeaminase activities reside on a single bifunctional protein (Tabor and Wyngarden, 1959; Drury et al., 1975).

1.1.3.2 The Interconversion of Folate Derivatives

For reviews of this topic, the reader is referred to MacKenzie (1984) and Appling (1991). Reactions which involve one-carbon transfers are dependent upon the ability of the organism to vary the oxidation state of the carbon unit attached to tetrahydrofolate. Regulation of the interconversion allows the cell to divert the limited supply of the folate coenzymes to satisfy the most immediate requirements of the cell (Appling, 1991). Folate derivatives at the formyl, methenyl and methylene oxidation levels are freely interconvertible, catalyzed reversibly by the enzymes methenyltetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase (Figure 2, reactions 9 & 10):

5,10-methenyl-H_4PteGlu + H_2O <=> 10-formyl-H_4PteGlu + H^+ (9)

5,10-methylene-H_4PteGlu + $NADP^+$ <=> 5,10-methenyl-H_4PteGlu + NADPH (10)

Therefore, C_1 units can enter the folate pool at more than one oxidation level and be available for folate-dependent syntheses. The formyltetrahydrofolate synthetase (see section 1.1.3.1), cyclohydrolase and dehydrogenase are associated on a single trifunctional protein in mammalian tissues (Paukert et al., 1976; Tan et al., 1977; Barlowe and Appling, 1988), named the C_1-THF synthase (Figure 2, reaction 8-10). In yeast, both the cytoplasmic and mitochondrial isozymes are trifunctional and are encoded by the ADE3 and MIS1 genes respectively (Appling and Rabinowitz, 1985a; Shannon and Rabinowitz, 1988). C_1-THF synthase consists of two separate domains, each containing a folate-binding site. One domain contains the dehydrogenase/cyclohydrolase activities and the other the synthetase activity (Tan and MacKenzie, 1977; Cohen and MacKenzie, 1978; Villar et al., 1985).

Trifunctional C_1-THF synthases possess NADP-dependent

methylenetetrahydrofolate dehydrogenase activity. Recent studies revealed an independent cytoplasmic NAD-dependent methylenetetrahydrofolate dehydrogenase enzyme (Table 2, reaction 10b) found only in embryonic, undifferentiated, or transformed cells as a monofunctional protein or as a bifunctional protein with methenyltetrahydrofolate cyclohydrolase (Mejia and MacKenzie, 1988; Shane, 1989). One suggested role for this enzyme was to increase the one-carbon flux into purine biosynthesis and away from other one-carbon cycles such as methionine synthesis during periods of increased synthesis.

1.1.3.3 One-Carbon Units and Biosyntheses *De Novo*.

Purine Synthesis Enzymes .

One-carbon units from 10-formyltetrahydrofolate can be added to the #8 and #2 carbons respectively of the purine ring by glycinamide ribonucleotide (GAR) transformylase and 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase (Figure 2, Table 2, reactions 12 and 13; Smith et al., 1981; Hartman et al., 1956; Hartman and Buchanan, 1959):

$$\text{10-formyl-}H_4\text{PteGlu} + \text{GAR} \text{ ------> } H_4\text{PteGlu} + \text{formyl-GAR} \qquad (12)$$

$$\text{10-formyl-}H_4\text{PteGlu}_n + \text{AICAR} \Rightarrow H_4\text{PteGlu} + \text{formyl-AICAR} \qquad (13)$$

Reaction 12 is irreversible; reaction 13, although reversible, favours formyl-AICAR formation (Brody et al., 1984). It was originally reported that 5,10-methylene-tetrahydrofolate donated the formyl group to glycinamide ribonucleotide (GAR) to give N-formylglycinamide ribonucleotide (formyl-GAR; Hartman et al., 1956). More recently, Smith et al. (1981) suggested that 10-formyltetrahydrofolate was the formylating agent, and that original experiments showed inactivity of 10-formyltetrahydrofolate due to the presence of the d-10-formyltetrahydrofolate derivative in racemic substrate mixtures. The ability to utilize 5,10-methenyl-tetrahydrofolate as a substrate was shown to arise from the association of GAR transformylase with the trifunctional C_1-THF synthase enzymes,

converting 5,10-methenyl- to 10-formyltetrahydrofolate (Smith et al., 1981).

Reaction 13 also appears to be involved in the synthesis of histidine. A branched pathway forms histidine in one direction and regenerates ATP in the other direction. The donation of C_1 units from 10-formyltetrahydrofolate appears to be involved in regeneration of ATP (for a review see Blakley, 1969).

Methionine Cycle.

Methionine synthesis (reviewed by Matthews, 1984) involves the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Figure 2 reaction 11) followed by the transfer of the methyl group to homocysteine to form methionine and to regenerate $H_4PteGlu_n$ (Figure 2, reaction 14). 5,10-methylenetetrahydrofolate reduction is catalyzed by the flavoprotein methylenetetrahydrofolate reductase as follows:

$$\text{5,10-methylene-}H_4\text{PteGlu}_n + \text{NADPH+H}^+ \Rightarrow \text{5-methyl-}H_4\text{PteGlu}_n + \text{NADP}^+ \quad (11)$$

Formation of 5-methyl-H_4PteGlu (Figure 2, reaction 11) is, in general, an irreversible process in vivo (Green et al., 1988) and is the committed step in methionine synthesis. Removal of the 5-methyl group can be achieved only through transmethylation via methionine synthase (Katzen and Buchanan, 1962; Brody et al., 1984).

The "methyl trap" hypothesis, advanced by Herbert and Zalusky (1962) and Noronha and Silverman (1962) postulates that decreased methionine synthesis causes a large amount of folate to be "trapped" as the 5-methyl derivative. In cases where 5-methyltetrahydrofolate formation exceeds transmethylation, a build-up of 5-methyltetrahydrofolate causes a decrease of nonmethylated forms of folate. These then cannot function in other folate-dependent reactions and a folate deficiency affects C_1 metabolism. An exception to this hypothesis occurs in some bacterial species that allow oxidation of 5-methyl-H_4PteGlu and a re-entry to the folate pool (Shane and Stokstad, 1977).

Methionine synthase catalyzes the transfer of the methyl group from 5-methyl-

$H_4PteGlu_n$ to homocysteine, as follows:

5-methyl-$H_4PteGlu_n$ + homocysteine => $H_4PteGlu_n$ + methionine (14)

Two methionine synthase enzyme systems exist, one cobalamin-dependent (that can use mono- and/or polyglutamate folates) and one cobalamin-independent (that can use only polyglutamate folates). E. coli expresses both types of methionine synthase (Banerjee and Matthews, 1990; Banerjee et al., 1990). The enzyme of mammalian cells and some bacteria contain only the cobalamin-dependent system (Taylor and Weissbach, 1973; Matthews, 1984). Higher plants and fungi have methionine synthases that are cobalamin-independent and folylpolyglutamate-dependent (Cossins, 1987), with folylpolyglutamate deficiency causing an inability to form methionine (Cossins and Chan, 1984; Chan and Cossins, 1984).

Thymidylate Cycle

The synthesis of thymidylate (Figure 2, reaction 15) involves a cyclic folate-dependent sequence of reactions catalyzed by thymidylate synthase (Table 2, reaction 15), and dihydrofolate reductase (Table 2, reaction 3) .

Thymidylate synthase (TS), localized in the nucleus (Brody et al., 1984), catalyzes the transfer of formaldehyde from folate to the C-5 of deoxyuridine monophosphate to form deoxythymidine monophosphate as follows:

5,10-methylene-$H_4PteGlu_n$+dUMP => $H_2PteGlu_n$+dTMP (15)

Hydrogen from the pteridine ring of $H_4PteGlu_n$ provides the reducing component for the reduction of the transferred one-carbon moiety and $H_4PteGlu$ is oxidized to $H_2PteGlu$ in the process (Blakley, 1969). 7,8-$H_2PteGlu_n$ (dihydrofolate) is then reduced to 5,6,7,8-$H_4PteGlu_n$ (tetrahydrofolate) in a reaction catalyzed by the enzyme dihydrofolate reductase (DHFR; Table 2, reaction 3):

$H_2PteGlu+NADPH+H^+ => H_4PteGlu+NADP^+$ (3)

The reader is directed to a review by Blakley (1984) for information on the general properties of this enzyme. A multienzyme complex (termed a replitase), containing all of the enzymes required for the synthesis of dNTPs (including TS and DHFR), channels ribonucleotides directly to replication forks during DNA synthesis (Reddy and Pardee, 1980, 1982; Chiu et al., 1982). TS and DHFR have received extensive attention as clinical targets, since cancer cells require larger amounts of thymidylate in the S phase of the cell cycle, and since low levels of both enzymes were found in non-proliferating tissues (Reddy, 1982; Rosowsky et al., 1992). Since the 1940's, the classical chemotherapeutic agents methotrexate (MTX) and aminopterin have been clinically used as powerful inhibitors of folate-requiring enzymes (reviewed by Blakley, 1984; Alt et al., 1978).

Formylmethionyl-tRNA (fMet-tRNA) Formation

Initiation of protein synthesis in bacteria and eukaryotic organelles require N-formylmethionyl-tRNA (Staben and Rabinowitz, 1984). This is in contrast to the cytosol of eukaryotes where initiation requires methionyl-tRNA (Tzagaloff, 1982). The enzyme catalyzing the formation of fmet-tRNA from met-tRNA is 10-formyl-tetrahydrofolate:methionyl-tRNA transformylase (Figure 2, Table 2, reaction 16), abbreviated transformylase.

$$10\text{-HCO-}H_4\text{PteGlu}_n + \text{met-tRNA} \Rightarrow \text{fMet-tRNA} + H_4\text{PteGlu}_n$$

The role of the transformylase, the sources of partial purification, and other factors involved in the initiation of protein synthesis in prokaryotes and eukaryote organelles, are discussed in a review by Kozak (1983).

1.1.3.4 Disposal of One-Carbon Units.

Folic acid deficiency impairs oxidation of formate to CO_2. This led to the proposed mechanism for the oxidation of excess one-carbon units to CO_2 (reviewed by Shane, 1989 and Blakley, 1969) in reactions catalyzed by:

a) 10-formyltetrahydrofolate:NADP oxidoreductase

$$10\text{-formyl-}H_4PteGlu + NADP^+ + H_2O \Rightarrow H_4PteGlu + CO_2 + NADPH+H^+$$

and b) 10-formyltetrahydrofolate deacylase

$$10\text{-formyltetrahydrofolate} + H_2O \Leftrightarrow \text{formaldehyde} + \text{tetrahydrofolate}$$

As mammalian tissue levels of 10-formyltetrahydrofolate are below their K_m values for the oxidoreductase (Scrutton and Beis, 1979), an elevation in level of 10-formyltetrahydrofolate should increase the rate of oxidation of C_1 units to CO_2 (Krebs et al., 1976). Evidence suggests that the oxidoreductase and deacylase activities can occur simultaneously as a bifunctional protein (Rios-Orlandi et al., 1986).

1.2 The Role of Folylpolyglutamates in Cells and Tissues

1.2.1 Tissue Folylpolyglutamates

The types and quantity of folate derivatives in tissues have been studied in a number of ways. The analytical procedures used can be divided into two categories: analysis of intact folates or analysis of one portion of the folate molecule, depending on whether one needs to know the identities of the one-carbon unit, the reductive state of the folate, the polyglutamate chain length of the folate, or all of the above. The methods used also depend on the heterogeneity of the unknown folates. Examples of analytical methods for determining types and quantities of folates are listed in Table 3.

Polyglutamyl folate derivatives are the biologically-active participants in the physiological reactions of one-carbon metabolism and not just storage forms of folate (Moran et al., 1976). The nature of folylpolyglutamates that predominate in different species and tissues have been extensively documented. They have been detected in microorganisms, fungi, plants and mammals; in many mammalian organs such as liver, intestine, kidney, brain and spleen; and in physiological fluids such as milk, cerebrospinal

fluid, bile, egg yolk and blood serum (Blakley, 1969; Cossins, 1984). Folylpolyglutamates have even been detected in the capsid of T4 bacteriophage for functioning of the phage tail (Kozloff and Lute, 1965). Intracellular folates isolated from natural sources were almost exclusively folylpolyglutamate derivatives with the glutamate moieties linked via γ-glutamyl peptide bonds (Figure 1). The occurrence of folates is discussed in greater detail by Baugh and Krumdieck (1971), Scott and Weir (1976), Krumdieck et al. (1983), Cossins (1980, 1984), Shane (1989) and Zheng et al. (1992).

Within any particular source, a single polyglutamate chain length predominates, although a distribution of glutamate chain lengths is usually observed (Brody et al., 1984). Exceptions to this rule include Clostridium, Bacillus subtilis, and Corynebacterium, where almost all of the total pool is of a particular chain length (Cossins, 1984). Within a single species, the distribution of polyglutamate chain lengths can differ between tissues (Brody et al., 1976). Indirect evidence from studies on antifolates have exploited these differences in polyglutamate formation between tumor tissue and normal tissue to improve effectiveness of chemotherapy (Jansen et al., 1992).

Most folylpolyglutamates appear to be located primarily in the cytoplasm and mitochondria (Selhub, 1990). Most intracellular folates may be bound to various soluble and membrane-bound folate-binding proteins under physiological conditions, and this is likely to modify the availability of folate substrates and inhibitors (Wagner, 1986). Isolated rat liver mitochondrial folate-binding proteins were observed to contain bound pentaglutamate. This suggests that bound folate forms were shown to be predominantly the pentaglutamate in rat liver mitochondria. Since the cytosol also contained primarily the pentaglutamate, the mitochondria and cytosol therefore have similar polyglutamate distributions (Shane, 1989).

Various media supplements cause changes in C_1 substitution and polyglutamyl chain length. L. casei studies show that nutritional folate concentrations affect glutamate chain length (Shane and Stokstad, 1975), such that the higher the external folate

Table 3 - Analytical Methods for Determining Types and Quantities of Folates (Cossins, 1984 with modifications).

Assay Technique	Method and Use	Detail
1. Differential Microbiological Assay (Cossins, 1984; Mullins and Duch, 1992)	Based on differential growth of assay organisms to various folates relative to standard growth curves. The amounts and types of folates present are measured by turbidity on a colorimeter, titration of lactic acid or reduction of 2,3,5-triphenyl tetrazolium chloride	*Lactobacillus casei* - grows on all folate forms up to Glu_3 *Streptococcus faecalis* - grows on non-methylated Glu_1 folates *Pediococcus cerevisiae* - grows on highly reduced, non-methylated Glu_1 derivatives.
2. Competitive / Noncompetitive Radioassay Techniques (Waxman and Schreiber, 1977)	Used for diagnosis of folate deficiency, especially when data obtained from microbiological assay can be seriously affected by antibiotics and folate antagonists.	Radioimunoassay; labeled and unlabeled folates (ligand) compete for a limited number of binding sites on a folate-binding protein.
3. Paper and Thin Layer Chromatography (Mullins and Duch, 1992)	Recently replaced by ion-exchange chromatography and HPLC	
4. Size Exclusion Chromatography (Shin, Williams and Stokstad, 1972)	Used to separate the various folate mono- as well as polyglutamated derivatives	Sephadex G-10, G-15, and G-25
5. Ion-Exchange Chromatography (Taylor and Hanna, 1977; Cossins and Chan, 1984)	Separates the mono- and polyglutamate forms of the folate cofactors. Gradient elution of from DEAE-cellulose has been used as a method for assay product analysis of polyglutamates	DEAE and TEAE celluloses, DEAE Sephadex, and QAE Sephadex
6. Affinity Chromatography (Ghitis, 1967)	Purifies folate quantitatively in tissue extracts using high-affinity folate-binding protein	
7. HPLC (Moran and Colman, 1984; Shane et al., 1983; Chan et al., 1988)	HPLC analysis of polyglutamates has been studied with ion-exchange as either $PteGlu_n$ derivatives or as $pABAGlu_n$ derivatives. A more recent development has been the use of a chiral stantionary phase for the resolution of isomers at the C-6 position.	Ion exchange or reverse-phase chromotography

concentration, the lower the chain length. S. faecalis, on the other hand, tends to reflect a constant polyglutamate pool size of Glu_4 irrespective of external folate concentration. In Neurospora, media supplements also affect polyglutamate synthesis. Glycine supplements stimulated a three-fold increase in folate content attributable to increased synthesis of unsubstituted polyglutamates (Cossins et al., 1976). Methionine supplements reduced total folate levels in wild-type and polyglutamate-deficient mutants, and glutamate chain lengths of increased size were detected (Chan and Cossins, 1980). This was completely reversible upon transfer to unsupplemented media.

It has been suggested that nutritional and physiological changes may affect substrate availability for folylpolyglutamate synthetase (FPGS) or γ-glutamyl hydrolase (GGH). Previous studies with Chinese hamster ovary cells (Taylor and Hanna, 1977) have shown that lowering the concentrations of vitamin B_{12} and/or methionine cause decreased synthesis of folylpolyglutamates, but no decrease in FPGS enzyme levels. Vitamin B_{12} and/or methionine deficiency may lead to an accumulation of folate derivatives that are poor substrates for the FPGS enzyme i.e. 5-methyltetrahydrofolate (Shin et al., 1975; Shane et al., 1977b), and this may explain the impaired folylpolyglutamate synthesis and decreased folate retention (see section 1.2.3). The resulting change in cell folate content and folylpolyglutamate chain length could therefore affect metabolic regulation of C_1 metabolism. (Cichowicz et al., 1981).

1.2.2 Physiological Significance of Polyglutamates

Most folate-dependent enzymes have greater affinities for polyglutamates than for monoglutamates (reviewed in Cichowicz et al., 1981; Kisliuk, 1981; McGuire and Bertino, 1981; McGuire and Coward, 1984; Shane, 1989). Polyglutamate forms of folate in most cases have increased affinity and decreased K_m as substrates/inhibitors for folate-requiring reactions. Except for the cobalamin-independent methionine synthase, enzymes of C_1

metabolism have the capacity in vitro to utilize monoglutamate forms of folate, although not necessarily at optimal levels. However, in vivo studies show that AUXB1 cells which lack the ability to synthesize polyglutamates are also auxotrophic for end products of C_1 metabolism (McBurney and Whitmore, 1974a; Taylor and Hanna, 1975, 1977, 1979). Studies with serine hydroxymethyltransferse suggest that the major kinetic advantage of lengthening the polyglutamate chain is achieved with extension to the chain to a triglutamate, and that further chain extension has relatively minor effect (Matthews et al., 1982). The metabolic advantages of longer chain lengths are not clear (Shane, 1989).

There is evidence that folylpolyglutamates are important in the cellular retention of folate (Shane and Stokstad, 1975, 1976). AUXB1 mutant cells transport folates normally, but they have low folate concentrations because they cannot retain folates (McBurney and Whitmore, 1974a). Consequently, metabolism of pteroylmonoglutamates to polyglutamate forms allows the cell to concentrate folates at much higher levels than in the external medium (Shane and Stokstad, 1983). Folates in mammalian tissues are metabolized to polyglutamates of chain lengths considerably longer than the triglutamate required for folate retention, but the metabolic advantages of this again are not clear (Shane, 1989).

The polyglutamate tail may allow substrate channeling between multifunctional protein complexes (Shane, 1989). The presence of more than one activity on a protein may offer a kinetic advantage if the intermediate substrate is not released from the protein, increasing substrate availability. Examples of such complexes are the formiminotransferase/cyclodeaminase complex, the C_1-THF synthase, and the DNA replitase. The optimal chain length for channeling of pig liver formiminotransferase/cyclodeaminase was different from the endogenous polyglutamate chain length of pig liver (MacKenzie and Baugh, 1980; Paquin et al., 1985). The regulatory significance of this finding is unknown.

1.3 Folylpolyglutamate Synthesis and Degradation

1.3.1 Folylpolyglutamate Synthetase

Over the last few years, a considerable amount of information has been obtained on the mechanism by which folylpolyglutamates are synthesized and the manner in which this synthesis is regulated. In early studies of bacterial FPGS, Masekar and Brown (1975) examined partially purified E. coli extracts, and these preparations catalyzed the ATP-dependent addition of L-glutamate to reduced pteroylmonoglutamate to form a diglutamate product. The enzyme folylpolyglutamate synthetase (tetrahydrofolate:L-glutamate γ-ligase (ADP forming); FPGS; E.C. 6.3.2.17) catalyzes the sequential ATP-dependent addition of glutamic acid residues to the folate molecules as follows:

$$H_4PteGlu + nATP + n\ glutamate \text{ -----> } H_4PteGlu_{n+1} + nADP + nPi \quad (4)$$

The enzymes from various sources studied are labile and activities are usually very low. Most synthetase activity has been localized in the cytoplasm, although some activity has been seen in mitochondrial fractions (Gawthorne and Smith, 1973; McGuire et al., 1979; Cossins and Chan, 1983, 1985; McDonald et al., 1995). The FPGS enzyme of bacteria, fungi and mammals has been studied in some detail, whereas plants have received limited study. In bacteria and mammalian tissues, polyglutamate synthesis is achieved by the action of a single enzyme that adds glutamate sequentially until the native polyglutamate length is reached (McGuire and Coward, 1984).

FPGS has been purified from several bacteria, including the purification to homogeneity of the Corynebacterium sp. (Shane, 1980a,b,c), L. casei (Bognar and Shane, 1983) and E. coli (Bognar et al., 1985) enzymes. The E. coli gene has also been cloned and sequenced (Bognar et al., 1985, 1987). Bacterial FPGS enzymes differ markedly from their eukaryotic counterparts in terms of substrate specificities, and in certain cases, in terms of their association with DHFS. E. coli and Corynebacterium sp.

FPGS possesses DHFS activity on the same protein (Bognar et al., 1985; Shane, 1980; Ferone and Warskow, 1983), an activity not possessed by eukaryotic enzymes studied (Ikeda and Iwai, 1970; Imeson and Cossins, 1991; McDonald et al., 1995). E. coli also contains a separate FPGS-like activity that can add additional glutamate moieties in α-peptide bond linkage to pteroyltriglutamates (Ferone et al., 1986a,b). It is not known whether other prokaryotic organisms also contain glutamates linked through the α-carboxyl group. The accumulation of pteroyltriglutamates in AUXB1-folC transfectants suggests that mammalian cells lack this activity.

Studies of FPGS structure and function were primarily based on mammalian systems, as folates and antifolates play an important clinical role in areas such as cancer therapy. The synthetase activity seems to be highest in liver, but appreciable levels seem to be found in most tissues, except muscle tissue (McGuire et al., 1979; Moran and Colman, 1984b). After preliminary characterization of crude enzyme preparations from sheep liver (Gawthorne and Smith, 1973) and partially purified enzyme from Chinese hamster ovary cells (Taylor and Hanna, 1977, McGuire et al., 1980) the enzyme was purified 70-fold from rat liver. This enzyme has been characterized from the liver of rat (McGuire et al., 1980), mouse (Moran and Colman, 1984), beef (Pristupa et al., 1992), and human (Clarke and Waxman, 1987), as well as from Chinese hamster ovary cells (Taylor and Hanna, 1977). The pig liver enzyme has been purified to homogeneity, at an enrichment of ca. 50,000-fold (Cichowicz and Shane, 1987a,b). This purified FPGS enzyme metabolizes tetrahydrofolate to the hexaglutamate in vitro, suggesting that a single enzyme is responsible for the conversion of folates to pteroylpolyglutamate forms. All mammalian FPGS enzymes have native molecular weights of 60,000-70,000, alkaline pH optima, absolute ATP-dependence, absolute monovalent cation requirement, and prefer tetrahydrofolate or methylenetetrahydrofolate substrates over other C_1 substituted folates (McGuire and Coward, 1984). Examples of other optimal FPGS substrates are 10-formyl-tetrahydrofolate (E. coli), 5,10-methylenetetrahydrofolate (L. casei) 5,10-methylene-

tetrahydrofolate (Neurospora), tetrahydrofolate (most mammalian tissues), and 5,10-methylenetetrahydrofolate (beef/mouse liver) (Cossins, 1984).

The FPGS of plants has been examined using pea cotyledons (Chan et al., 1986; Imeson et al., 1990). When FPGS activity was examined during early seedling development, the enzyme was found to be present in the mature, quiescent seed, and additional FPGS synthesis was not detected immediately after germination. The properties of the synthetase were similar to those reported for bacterial and mammalian systems. However, the optimal folate substrates tetrahydrofolate and/or 5,10-methylenetetrahydrofolate utilized by this enzyme vary from other FPGS enzymes.

FPGS has broad specificity and can add glutamates to antifolates as well as folates (Cossins, 1984). Many cytotoxic folate analogues, after entering cells, become polyglutamated, and this may affect their inhibitory activities, selectivity, toxicity, cellular retention and duration of action (Kalman, 1990; Schirch and Strong, 1989).

1.3.2 γ-Glutamyl Hydrolase

In mammals, folate is obtained from the diet or from intestinal flora (Stryer, 1988). Dietary folates, primarily polyglutamates, are impermeable to cellular membranes as seen in section 1.2.3. Entry of folates into the gut requires hydrolysis of folylpolyglutamates prior to absorption via a high-affinity carrier-mediated mechanism (Henderson, 1986) that can transport folate only in the monoglutamate form. Two separate classes of γ-glutamyl hydrolase activities were discovered (Reisenauer et al., 1977), also known as pteroylpolyglutamate hydrolases or folate conjugases. Most conjugases studied tend to be lysosomal in origin (γ-glutamyl-x carboxypeptidase; EC 3.4.19.9), are endopeptidases and have acidic pH optima (Silink et al., 1975; Priest et al., 1982; Wang et al., 1986). A second type of conjugase is the microsomal GGH (pteroylpoly-γ-glutamate carboxypeptidase; EC 3.4.19.8), typical of human jujenal brush border membranes, which acts as an exopeptidase at neutral pH (McGuire and Coward, 1984). Once monoglutamate

derivatives are formed and transported into cells, they can then be reconjugated by folylpolyglutamate synthetase as seen in section 1.3.1.

1.3.3 Folylpolyglutamate Mutants

Studies of this enzyme have drawn attention to mutations of the FPGS gene that have resulted in polyglutamate deficiencies. The Chinese hamster ovary mutant strain AUXB1 demonstrates the physiological importance of polyglutamates and folylpolyglutamate synthesis. These mutants require exogenous glycine, thymidylate, and a purine for growth (McBurney and Whitmore, 1974a,b). The mutant cells also have an absolute requirement for supplemental methionine, whereas the wild-type line can be cultured in the absence of methionine, provided that sufficient vitamin B_{12}, folate, and homocysteine are provided. The mutant cells contain low intracellular folate levels, all of which appear to be pteroylmonoglutamate. Normalizing intracellular folate levels by increasing the medium folate content does not eliminate the mutant phenotype, even though folate transport appears normal. This indicates that the phenotype is due to a lack of folylpolyglutamates rather than low intracellular folate levels (Sussman et al., 1986). The mutant phenotype was confirmed to be due to a lack of detectable folylpolyglutamate synthetase activity (Taylor and Hanna, 1977; 1979) and that AUXB1 cells contain pteroylmonoglutamates, while hexa- and heptaglutamates comprise over 95% of intracellular folates in wild-type CHO cells (Foo and Shane, 1982). There is no information to date to identify the AUXB1 mutation, although the reversion frequency of AUXB1 was consistent with a single genetic mutation. AUXB1 cells lacked both cytoplasmic and mitochondrial folylpolyglutamates.

In E. coli, the SF4 mutant was observed to have reduced FPGS and DHFS activities, both of which were encoded by the folC gene. The mutants require glycine and methionine supplementation when grown on minimal media (Bognar et al., 1985, 1987). Although the mutant was leaky, it exhibited a dependence for methionine that was sufficient

for selection during cloning the wild-type gene. The effects of the mutation caused the loss of FPGS activity, and resulted in low levels of intracellular folylpolyglutamates.

Some studies with the mutants described involve transformation/transfection of mutant cell lines to clone FPGS genes by functional complementation. Thus various wild-type FPGS genes restore ability to express enzyme activity in mutant cell lines (Bognar et al., 1985; Sussman et al., 1986; Garrow et al., 1992; Garrow and Shane, 1993; Osborne et al., 1993) and folylpolyglutamates are synthesized in vivo (Osborne et al., 1993; Shane et al., 1993; Lowe et al., 1993). E. coli FPGS has also been the subject of site-directed mutagenesis. The difference in sequence between the SF4 mutant and wild-type folC genes have identified the location of the mutation to be a G to A base change at nucleotide 925 resulting in an A to T amino acid change at amino acid 309. Using functional complementation of SF4 and folC deletion mutants, in vitro mutagenesis was performed to alter codon 309 to code for other amino acids to determine mutational effect (Bognar et al., 1987; Keshavjee et al., 1991). The results confirm that a G to A change at position 925 is the site of the mutation in strain SF4.

1.4 The Present Study of Neurospora crassa Folylpolyglutamate Synthetase

Polyglutamate synthesis in Neurospora is affected by nuclear mutations at the met-6, met-s2706 and mac loci (Selhub, 1970; Sakami et al., 1973; Ritari et al., 1973a,b; Cossins and Chan, 1983; 1984; 1985) of chromosome 1R. These mutants fail to generate polyglutamates in vivo and display methionine auxotrophic phenotypes. Met-6 mutant appears to be stimulated by methionine, mac appears to be stimulated by additional adenine and cysteine supplementation, but only methionine appears to be an absolute requirement. Met-s2706 appears quite leaky, producing significant mycelia at a reduced rate in minimal media, but producing mycelia at wild-type rates when supplemented with methionine.

In early studies with Neurospora crassa (Selhub, 1970; Cossins and Chan, 1983), folylpolyglutamate compositions in the wild-type and the methionine auxotrophic strain

folylpolyglutamate compositions in the wild-type and the methionine auxotrophic strain met-6 differed markedly. Differential microbiological assay and DEAE-cellulose chromatography before and after hydrolase treatments showed that nearly 95% of Neurospora wild-type folate pools consisted of long-chain 5-methyltetrahydrofolate polyglutamates (Selhub, 1970). Using the same techniques, the met-6 mutant strain was shown to contain primarily unsubstituted monoglutamyl folates (Selhub, 1970; Selhub et al., 1972). Met-6 and mac clearly lacked hexaglutamyl folates, as judged by chromatography of labeled azodye derivatives and conjugase treatments of mutant extracts. Met-s2706 contained 10% of the total wild-type folylpolyglutamates (Cossins and Chan, 1985). In the three mutants, the polyglutamate deficiencies appear to be caused by mutations affecting expression of FPGS.

Biochemical and genetic evidence suggested that polyglutamate synthesis may involve more than one synthetase activity (Selhub, 1970; Cossins and Chan, 1984). The first activity, detected in the met-6 mutant (Cossins and Chan, 1984), adds a glutamate residue to folate to produce folyldiglutamates (reaction 4A). The second activity, found in the mac and met-s2706 mutants (Cossins and Chan, 1984), involves subsequent additions of glutamates to form longer chain folylpolyglutamates from $H_4PteGlu_2$ (reaction 4B).

$$H_4PteGlu + ATP + L\text{-glutamate} \rightarrow H_4PteGlu_2 + ADP + Pi \qquad (4A).$$

$$H_4PteGlu_2 + 4ATP + 4L\text{-glutamate} \rightarrow H_4PteGlu_6 + 4ADP + 4Pi \qquad (4B).$$

Earlier studies have shown that met-6 could only form diglutamates in vitro and was unable to use the diglutamate to form longer chain length folates (Table 4; Cossins and Chan, 1984). On the other hand, mac used the diglutamate substrate in vitro but was unable to add glutamate to the monoglutamate substrate (Table 4; Cossins and Chan, 1984). In vitro, the major FPGS product of the wild-type was hexaglutamyl folate (Cossins and Chan, 1984). Differential heat treatments and fractionation of cytosolic proteins provided evidence that these partial FPGS activities (4A) and (4B) were present in the wild-type.

and formed long-chained polyglutamates in vivo. It has therefore been suggested that two enzymes could be responsible for production of the hexaglutamate found in this species.

In common with the mitochondria of other species, N. crassa mitochondria possess FPGS activity (Table 4; Cossins and Chan, 1984). Wild-type mitochondrial FPGS appears to produce a triglutamate folate from a diglutamate substrate (Cossins and Chan, 1984), an activity which precipitates with ammonium sulphate at 0-35% of saturation. The met-s2706 mutant has a mutation affecting the cytosolic, tetrahydrofolate diglutamate-forming activity (equation 4A), similar to the mac mutant (Cossins and Chan, 1985). However, mitochondria of the met-s2706 mutant are able to synthesize polyglutamates (Table 4). Neither met-6 nor mac have detectable mitochondrial FPGS activity (Table 4 ; Cossins and Chan, 1984).

The genetic relationship between met-6 and mac remains unclear. The three methionine mutants, met-6, mac and met-s2706, map to the met-6 region. Data from Murray (1968 & 1969) clearly place the mutants in order thi-1 - mac - met-s2706 - met-6 - ad-9. Both mac and met-s2706 complement met-6, but mac and met-s2706 do not complement each other, indicating allelism between mac and met-s2706 (Murray, 1969; Cossins and Chan, 1984). On the other hand, in high resolution recombination studies, met-6 and met-s2706 behaved as alleles of the same gene, while mac appeared to be very closely linked (Murray, 1968 & 1969). Thus, the two FPGS activities observed in the mutants might define different genes or separate functional domains of the same gene. It is also not clear whether these FPGS activities occur in the wild-type as distinct proteins.

The present research focuses on certain aspects of the biochemistry and molecular biology of the enzyme FPGS in Neurospora crassa. This research using the three folate-related mutants has the potential to further our understanding of the basic mechanism of FPGS action. The first approach was to clone and sequence the met-6^{+} gene in order to gather information about the gene product. Once the met-6^{+} gene was identified, its coding

Table 4. Folylpolyglutamate Synthetase Activities After $(NH_4)_2SO_4$ Fractionation of Mycelial Extracts.*

Strain	Folate substrate	Cytosolic activity	Mitochondrial activity
wild type	$H_4PteGlu_1$ (mono)	+	-
	$H_4PteGlu_2$ (di)	+	+
met-6	$H_4PteGlu_1$	+	-
	$H_4PteGlu_2$	-	-
mac	$H_4PteGlu_1$	-	-
	$H_4PteGlu_2$	+	-
met-s2706	$H_4PteGlu_1$	-	-
	$H_4PteGlu_2$	+	+

* data from Cossins and Chan, 1984; 1985

(+) denotes detectable activity based on the incorporation of tritiated glutamate

(-) denotes that FPGS activity, based on the incorporation of tritiated glutamate was not significantly greater than observed in controls lacking mycelial protein.

sequence was confirmed by sequencing and open reading frame analysis. The second approach was to examine the FPGS enzyme activities before and after transformation. As an extension of earlier work, in which the wild-type protein was purified to near homogeneity (Chan et al., 1991), partial purification of the mutant and transformant enzymes was performed to provide information on the nature of the proteins that produce the products shown in equations 4A and 4B respectively.

2. Materials and Methods

2.1 Chemicals

Reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and from the Edmonton offices of Fisher Scientific, Baxter-Canlab, BDH Chemicals, and Terochem. Pteroyldiglutamate and p-aminobenzoylpolyglutamate (n=1-7) markers were obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. [U-^{3}H]L-glutamate, obtained from Amersham-Searle Corporation, Arlington Heights, Illinois, was diluted with carrier L-glutamate to give a specific radioactivity of 25 µCi/µmol. Sequenase™ DNA sequencing kits were also obtained from Amersham-Searle. Sequencing primers were purchased from New England BioLabs, Inc. or were prepared by DNA Synthesis Laboratory, Department of Biological Sciences, University of Alberta. Ring labeled [^{14}C]p-ABA, purchased from Sigma, was dissolved in ddH_2O to give a final concentration of 100 µCi/ml with a specific radioactivity of 6.8 mCi/mmol. [^{14}C]$PteGlu_3$, labeled in the terminal glutamate moiety, was supplied by Dr. Carlos Krumdieck, Department of Biochemistry, University of Alabama, Birmingham, and was diluted with carrier $PteGlu_3$ to give a specific activity of 0.0033 µCi/µmol. BioGel P2 (200-400 mesh) was purchased from Bio-Rad Laboratories, Richmond, California. Whatman DE-52 anion exchange cellulose and Whatman Partisil 10 SAX HPLC columns were purchased from Rose Scientific, Edmonton, Alberta. Sephadex G-200 was purchased from Pharmacia, Montreal, Quebec. Heparin-Agarose, Reactive Green 5, and molecular weight marker proteins were supplied by Sigma. Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL) Inc., BMC or New England BioLabs, Inc. Protein measurement was based on the method of Bradford (1976) using Bio-Rad Protein assay kits. Bray's scintillation fluor (Bray, 1960) was prepared from scintillation grade chemicals supplied by Sigma Chemical Co. and Fisher Scientific.

2.2 Strains and Culture Conditions

Growth and handling of Neurospora crassa strains was as described in Davis and de Serres (1970). Three wild-type strains of N. crassa (Lindegren A, FGSC 853; 74-OR23-1A, FGSC 987; and Emerson - Em 5256, FGSC 424), three methionine-requiring mutant strains (met-6, FGSC 1330; mac, FGSC 3609; and met-s2706, FGSC 4248), as well as transformed mutant strains used in this study are described in Table 5. N. crassa cultures were maintained and grown in Vogel's defined minimal medium (Davis and de Serres, 1970; Cossins and Chan, 1984), with mutant cultures routinely receiving supplements of 0.2 mM L-methionine, and transformant cultures receiving 0.5 µg/ml benomyl. For generation of conidia, cultures on solid media were incubated at 30°C for approximately 72 hours, followed by exposure to light for conidiation. For generation of mycelia, cultures in liquid media were incubated at 30°C with aeration for 24 to 48 hours. Cells were harvested by vacuum filtration while growth was logarithmic, and the mycelial pad was washed with sterile cold ddH_2O.

All E. coli strains used are detailed in Table 6. Unless otherwise indicated, all E. coli strains were grown in Luria-Brufoni broth (L-broth; Lennox, 1955; Sambrook et al., 1989). Ampicillin (100 µg·ml^{-1}), tetracycline (20 µg.ml-1) and IPTG (25 µg·ml^{-1}) / X-gal (50 µg·ml^{-1}) were added to media as necessary for plasmid selection. Cosmid clones were maintained in E. coli strain DH1. The maintenance and utilization of the cosmid library was as described by Drygas et al. (1989). Bacterial plasmids and recombinant plasmid clones were maintained in E. coli strains JM 83 or XL1-Blue. λ-ZAP cDNA library amplification was performed in XL1-Blue. XL1-Blue host cells for λ-ZAP amplification were grown overnight in L-broth supplemented with 0.2% maltose and 10 mM $MgSO_4$.

Table 5. Neurospora crassa Strains Used in the Present Studies

Strain	Genotype	Source
74-OR23-1A	A; wild type	Oak Ridge; FGSC 987
Lindegren A	A; wild type	Lindegren; FGSC 853
Emerson	A; wild type	Abbott x Lindegren; FGSC 424
met-6	methionine auxotroph	FGSC 1330
mac	methionine auxotroph	FGSC 3609
met-S2706	methionine auxotroph	FGSC 4248
met-6 + pSV50 (m6-pSV50)	transformant control	see text
met-6 + 4:11:C (m6-T4:11)	cosmid transformant	see text
met-6 + 7:10:D (m6-T7:10)	cosmid transformant	see text
met-6 + pSV50/pIA50 (m6-TIA50)	plasmid transformant	see text
mac + pSV50 (mac-pSV50)	transformant control	see text
mac + 4:11:C (mac-T4:11)	cosmid transformant	see text
mac + 7:10:D (mac-T7:10)	cosmid transformant	see text
mac + pSV50/pIA50 (mac-TIA50)	plasmid transformant	see text
met-S2706 + pSV50 (mS27-pSV50)	transformant control	see text
met-S2706 + 4:11:C (mS27-T4:11)	cosmid transformant	see text
met-S2706 + 7:10:D (mS27-T7:10)	cosmid transformant	see text
met-S2706 + pSV50/pIA50 (mS27-TIA50)	plasmid transformant	see text

Table 6. E. coli Strains Used in the Present Studies

Strain	Genotype	Reference
DH1	F$^-$, recA1,endA1, gyrA96, thi-1, hsdR17 (r_K^-, m_K^+) supE44, relA1?, λ^-	Hanahan, 1983; Vollmer and Yanofsky, 1986
JM83	r-, ara, Δ(lac-proAB), rpsL, thi ϕ80, lacZΔM15, hsdR-	Vieira and Messing,1982; Yanisch-Perron et al., 1985.
XL1-Blue	endA1, hsdR17, (r_K^-, m_K^-), supE44 thi-1,λ-, recA1, gyrA96, relA1, (lac-), [F' proA$^+$B$^+$,lacI^q ZΔM15, Tn10(tet^r)]	Messing et al., 1983.

2.3 Construction of the Met-6$^+$ Recombinant Plasmid

Cosmid pSV50 (Vollmer and Yanofsky, 1986) and the plasmids pUC19 (Yannisch-Perron et al., 1986), pGem-T (Promega, Inc.), and pBluescript (Stratagene Inc.) were used as vectors in this study. The cosmid pSV50, derived from pBR322, contained the E. coli ampicillin-resistance gene (Amp^R), the N. crassa tub-2 ß-tubulin gene that confers benomyl resistance, and λ cos sites for cosmid library preparation .

Recombinant cosmids and plasmids are listed in Table 7. The N. crassa cosmid clones pSV50-4:11:C and pSV50-7:10:D (constructed in cosmid vector pSV50) were a gift from Dr. T. Schmidhauser, Southern Illinois University. The cosmid clone pSV50-4.1.3.1 was isolated from a pSV50 cosmid library of strain OR23-74-1A DNA present in our laboratory (Drygas et al., 1989). Cosmid pSV50-4.1.3.1, containing the met-6$^+$ gene, was screened by following the ICN Biomedical, Inc. protocol for colony lifts to Biotrans nylon membrane, using a portion of pSV50-4:11:C as a probe (see Section 2.5). Cosmid and plasmid isolations were performed according to the alkaline lysis procedure (Birnboim and Doly, 1979) and when necessary, large scale preparations were further purified by CsCl-ethidium bromide gradient centrifugation (Maniatis et al.,1982).

Cloning methods, restriction endonuclease digestion and ligations were performed according to manufacturer's instructions or as described by Maniatis et al (1982) or Sambrook et al. (1989). The 40 kb cosmid pSV50-4.1.3.1 was digested using various restriction endonucleases, and the individual fragments were used to transform met-6 mutant spheroplasts (see section 2.4). A 4.3 kb Eco RI/Pst I restriction fragment from the cosmid pSV50-4.1.3.1, capable of transforming met-6 mutant spheroplasts to prototrophy (see section 2.4), was excised and recovered from 0.8% submarine agarose gels (0.8% agarose, 0.1 M Tris-acetate pH 8.3, 2 mM EDTA; 0.0133 µg/mL ethidium bromide) using either electroelution (Maniatis et al., 1982) or sodium iodide/glass (Vogelstein and Gillespie, 1979). The DNA was resuspended in water and ligated into the compatible

Table 7. Recombinant plasmids

Plasmid	Parental plasmid	Description of the cloned fragment
pSV50-4:11:C	pSV50	an insert of *ca* . 40 kb carrying the met-6$^+$ gene isolated from *N. crassa* genomic library; used in transformant strains for folate analysis.
pSV50-7:10:D	pSV50	an insert of *ca* . 40 kb carrying the met-6$^+$ gene isolated from *N. crassa* genomic library; used in transformant strains for folate analysis.
pSV50-4.1.3.1	pSV50	an additional cosmid containing of the met-6$^+$ gene isolated from a *N. crassa* genomic library; used for subcloning.
pIA23	pUC19	an insert of ca. 4.5 kb carrying the met-6$^+$ gene from an EcoR1/Pst 1 fragment subcloned from pSV50-4.1.3.1.
pIA50	pUC19	an insert of ca. 3.5 kb carrying the met-6$^+$ gene from an EcoR1/BamH1 fragment subcloned from pIA23.
pIA62	pUC19	an insert of ca. 0.7 kb carrying a portion of the met-6$^+$ gene from an EcoR1/Kpn1 fragment subcloned from pIA50.
pIA68	pUC19	an insert of ca. 2.7 kb carrying a portion of the met-6$^+$ gene from an Kpn1/BamH1 fragment subcloned from pIA50
pcD21; pcD31	pBluescript	1.6 kb met-6$^+$ cDNA clones with EcoR1 linkers; first isolates; truncated 5' end.

cloning site of plasmid pUC19 to form plasmid pIA23 (see Results). E. coli transformation experiments were carried out following the calcium chloride procedure of Mandel and Higa (1970).

A 3.5 kb EcoRI/BamHI digested fragment of pIA23 was isolated as above and ligated into pUC19, producing the plasmid pIA50 (see Results). pIA50 was the plasmid used for further analysis of the met-6^{+} gene. The 3.5 kb EcoRI/BamHI fragment was digested using the enzyme KpnI to give two fragments, neither of which were unable to transform the met-6 mutant spheroplasts (see Results). Each fragment was ligated into plasmid pUC19, and the recombinant plasmids pIA62 and pIA68 are the EcoRI/KpnI (0.7 kb) and KpnI/BamHI (2.7 kb) fragments of the EcoRI/BamHI insert of pIA50, respectively.

2.4 Transformation of Neurospora Mutants to the Wild-Type Phenotype

The removal of cell walls from conidia in the preparation of N. crassa spheroplasts was performed using the technique of Schweizer et al. (1981) with the modifications described by Akins and Lambowitz (1985). All operations were performed under sterile conditions except for addition of Novozyme™ 234 (Novo Laboratories Inc.) or Lysis Enzyme (Sigma) powder. Mutant conidia were germinated for 7-8 hours to a minimum of 80% germination for spheroplast preparation. Spheroplast viability was tested using serial dilutions of the spheroplast suspension in regeneration agar supplemented with 0.2 mM methionine.

The mutant N. crassa strains met-6, mac, and met-s2706 were used as the recipient spheroplasts for transformation, except during subcloning where the met-6 mutant strain was used exclusively. The $CaCl_2$-mediated transformation procedure was as described by Akins and Lambowitz (1985). Cosmid clones were transformed alone; plasmid or linear DNAs were co-transformed with pSV50 DNA to provide benomyl resistance, assuring transformation and maintaining low background. Benomyl

transformation plates were incubated at 30°C for 36-48 hr. Individual transformed colonies isolated for further analysis from top agar plugs were cultured on slants of solidified Vogel's minimal medium containing benomyl (0.5 µg/l).

2.5 Southern Blot Analyses

N. crassa genomic DNA was isolated using a modification of the procedure of Schectman (1986). Mycelia was ground in 10 volumes 50 mM EDTA, 0.1% SDS, pH 8.0 in the presence of acid-washed sea sand. After heating the homogenate at 70°C for 15 minutes and cooling on ice for 10 minutes, 0.1 volumes of 8 M sodium acetate, pH 4.3, was added and the solution was incubated for 1 hour on ice. After centrifugation at 10,000 x g for 15 minutes, 1 volume of 2-propanol was added to the supernatant. A DNA clot was removed and resuspended in 1 mM EDTA, pH 8.0 and 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0. The suspension was extracted once each with phenol, phenol/chloroform and chloroform, and then ethanol precipitated in the presence of 0.3 M sodium acetate. The precipitate was washed with 70% ethanol and resuspended in ddH_2O.

10 µg aliquots of DNA from each strain were digested with the appropriate restriction endonuclease and run on an 0.8% submarine agarose gel. After partial depurination in 0.2 M HCl, denaturation in 0.5 M NaOH, 1.5 M NaCl and neutralization in 3 M sodium acetate, the gel was blotted to a Biotrans nylon membrane (ICN Biomedical, Inc.) following the procedure for Southern analysis modified from the procedure described by Southern (1979).

Restriction fragment or PCR amplification products (see section 2.6.1) used as hybridization probes were excised and purified from agarose gels using sodium iodide/glass (Vogelstein and Gillespie, 1979). Products were either uniformly labeled using random oligonucleotide primer extension in the presence of ^{32}P-dCTP, (Feinberg and Vogelstein, 1983; 1984), or non-radioactively labeled using digoxigenin labelling (BMC Inc. instructions). Southern blots were screened using labeled 2.7 kb KpnI-BamHI

fragments isolated from pIA68. Alternatively, 0.7 kb EcoRI-KpnI fragments were isolated from pIA62 as hybridization probes. To determine the incorporation of radioactivity into DNA, two 1 µl samples were spotted onto separate DE81 ion exchange Whatman filter papers; one paper was then washed with 50 mL of 0.3 M ammonium formate. After scintillation counting, the difference in counts on the unwashed and washed filters was used as a measure of the efficiency of incorporation of labeled nucleotides into DNA polymers.

Prehybridization, hybridization and washing of membranes were carried out at 42°C or 65°C according to the ICN protocol. Autoradiography was performed for 2 to 24 hours at -70°C in the presence of intensifying screens. XAR-5 (Kodak) or Fuji X-ray film was used for autoradiography and developed according to manufacturers' instructions.

2.6 Polymerase Chain Reaction (PCR)

Fragments of mutant and wild-type genomic DNA were generated by PCR for cloning into bacterial vector pGEM-T and for direct sequencing (see section 2.7) Oligonucleotide primers for PCR were originally synthesized as primers for sequence determination (see section 2.7).

For rapid small scale isolation of genomic DNA template, conidial DNA extracts were prepared. Small samples of conidia were suspended in sterile 1 M sorbitol, 20 mM EDTA, 3 mg/ml Lysing Enzyme (Sigma), followed by a 15 minute incubation at 37°C. The samples were pelleted in a microfuge and washed twice with 1 M Sorbitol, 20 mM EDTA before resuspension in ddH_2O. After boiling for 10 minutes, 3 µl of the resulting preparation were used per 30 µl PCR reaction as template. For large scale genomic DNA isolations, DNA was prepared using the procedure of Schectman (1986) with modifications as described in section 2.5.

Taq PCR amplification, utilized primarily for the generation of PCR products for sequence analysis (see section 2.7), involved approximately 2 ng/µl of template DNA in a

solution of 50 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.005% ß-mercaptoethanol, 0.1 µg/µl bovine serum albumin (BMC Inc.), 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1 ng/µl of each oligonucleotide primer, and 2.5 Units of Taq polymerase (BRL Inc.).

PCR utilizing VENT polymerase containing 3' to 5' proofreading function to minimize replication errors was utilized for the generation of DNA fragments for cloning. The reaction was performed using mixtures containing 2 ng/µl template DNA, 1X Vent buffer containing $MgCl_2$, 0.05 µg/µl bovine serum albumin (BMC Inc.), 0.2 mM dNTPs, 1 ng/µl of each oligonucleotide primer, and 1 Unit Vent polymerase.

Reactions were carried out in an AutoGene Thermal Cycler or a Stratagene Robocycler 40. The standard amplification program started with one cycle of 5 minutes at 95°C, 1 minute at 50-60°C and 4 minutes at 73°C, followed by 29 cycles of 1 minute at 92°C, 1 minute at 50-60°C and 4 minutes at 73°C. Amplification products were run on 0.8% agarose gels, excised and purified using sodium iodide/glass (Vogelstein and Gillespie, 1979).

When PCR products were to be cloned, pGEM-T (Promega, Inc.) was used as the cloning vector following standard methodology of the manufacturer. This linear vector had 3' overhanging T nucleotide ends, which permitted ligation between itself and a complementary 3' overhanging A nucleotide created in Taq-amplified products. This vector served for cloning VENT amplified products as well as TAQ PCR-amplified products, even though VENT PCR products theoretically were lacking the overhang A, associated with Taq-amplified product. The reason for this observation is unknown.

2.7 DNA Sequence Determination

All DNA sequencing, performed on double-stranded plasmids, was determined by the dideoxy chain termination method of Sanger et al. (1977) using Sequenase™ sequencing kits. The sequencing reactions were carried out using [α-^{35}S]dATP with

minor modifications to the Sequenase™ protocol. Following the annealing and sequencing protocol, the samples were heated in boiling water for 2 minutes to achieve denaturation. Samples were loaded immediately onto 5% (w/v) polyacrylamide gels and electrophoresed in 0.1 M Tris-borate pH 8.3, 2 mM EDTA. Gels were dried at 80°C in a Slab Dryer (BioRad) Model 483, and autoradiography performed at room temperature for 12 to 96 hours. XAR-5 (Kodak) or Fuji X-ray film was used for autoradiography. In regions where compressions were encountered, labelling and termination mixes containing dITP instead of dGTP were used to sequence the region (Sanger et al., 1982).

PCR amplification products were sequenced following the dideoxy chain termination method of Sanger et al. (1977) using Sequenase™ sequencing kits, with minor modifications to the manufacturer's instructions. PCR products (see section 2.6) were isolated from 0.8% agarose gels with sodium iodide/glass (Vogelstein and Gillespie, 1979) and resuspended in distilled water. Approximately 1-2 µg of DNA solution were combined with 100ng of a specific primer and heated to 100°C for 10 minutes. Annealing was carried out by immediate flash freezing in a dry ice/ethanol bath, where no annealing buffer was required. The sequencing reactions were then carried out with minor modifications of the Sequenase™ protocol described above. Volumes and concentrations were changed to accomodate the change in initial volume and the exclusion of enzyme dilution buffer. After completion of the reactions, the samples were electrophoresed as above.

2.8 RNA Characterization

2.8.1 Preparation of RNA

To inactivate RNAses, all glassware used for RNA isolation was baked at 180°C for 3 hours, and all solutions used were treated with 0.1% diethyl pyrocarbonate (DEPC) for a minimum of 12 hours prior to autoclaving. Mycelium from the wild-type strain 74-OR23-1A was harvested by filtration, washed in distilled water, and immediately frozen in liquid nitrogen. Total RNA was extracted by the procedure of Reinert et al. (1981) with

minor modifications as described by Nargang et al. (1988). Liquid nitrogen-frozen mycelium was ground to a powder with heat sterilized acid-washed sand (4 g sand per 1 g of mycelium). The powder was ground to a smooth paste with 2 volumes of guanidinium buffer (7.5 M guanidinium-HCl, 25 mM sodium citrate, 0.5% (w/v) lauryl sarcosinate and 0.1 M ß-mercaptoethanol). The suspension was centrifuged at 10,000 x g and protein was removed from the supernatant using two extractions with equal volumes of phenol/chloroform/isoamyl alcohol (25/24/1 by volume) and one extraction with an equal volume of chloroform/isoamyl alcohol (24/1). The final aqueous phase was mixed with an equal volume of isopropanol and 0.1 volume of 3M sodium acetate, left at -20°C overnight, and then centrifuged at 4°C for 15 minutes at 10,000 x g. The resulting pellet was resuspended in sterile TE buffer.

Poly(A) RNA was separated from non-polyadenylated RNA species by oligo(dT)-cellulose chromatography as described by Maniatis et al. (1982). 5 to 10 mg of total RNA was applied to 0.2 mg of hydrated oligo(dT)-cellulose (Pharmacia LKB Biotechnology Inc., type 7), packed in a disposable econocolumn (BioRad). Poly(A) RNA was stored at -70°C at a concentration of 1 µg/µl.

2.8.2 RNA Electrophoresis and Analysis

For Northern blots, 10-50 µg total RNA or 1-5 µg poly(A) RNA were denatured at 65°C for 15 minutes in denaturation buffer (1X MOPS buffer {0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0}, 9% formaldehyde, 7.5% glycerol, 0.5% (w/v) bromophenol blue, 50% deionized formamide). The RNA was then electrophoresed through 1.2% formaldehyde agarose gels (6% formaldehyde, 1X MOPS buffer). The RNA was transferred to nylon membrane (Biotrans, ICN Biomedical Inc.) by capillary transfer (Foury et al. described in Sambrook, 1989) using a sponge placed in a reservoir containing 10x SSC solution (solutions defined in Sambrook, 1989).

Following overnight transfer, the blot was baked in an oven at 80°C for 1 hour and hybridized to specific ^{32}P-containing DNA probes (addition of 10^6 dpm·cm^{-3} probe with a specific activity ≥ 10^8 dpm·μg^{-1} DNA), in a solution of 50% formamide, 5X SSC, 1% SDS, 5X Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA and 10% polyethylene glycol. Blots were hybridized at 42°C for 12 hours and washed twice in 2X SSPE at room temperature for 15 minutes each, twice in 2X SSPE at 65°C for 45 minutes each, and twice in 0.1X SSPE at room temperature for 15 minutes each. Blots were exposed to X-ray film for 2-168 hours at -70°C.

2.9 Isolation of Met-6$^+$ cDNA Clones

2.9.1 Isolation of cDNA Clones from the λ-ZAP Library

A N. crassa cDNA library, constructed in the λ-ZAP/EcoRI vector (Stratagene Inc.), was generously provided by Dr. Matthew Sachs, Stanford, California (Orbach et al., 1986). A second cDNA library studied was constructed in our laboratory. The cDNA library clones (5 x 10^5 pfu per plate; 10-150 mm plates) were lifted onto Biotrans nylon membranes (ICN Biomedicals, Inc.) following the protocol for Plaque lifts.

Products used for probes were uniformly radioactively labeled using random oligonucleotide primer extension in the presence of ^{32}P-dCTP (Feinberg and Vogelstein, 1983; 1984). These libraries were screened using the 2.7 kb KpnI-BamHI fragment from pIA68 or the 0.7 kb EcoRI-KpnI fragment from pIA62 as hybridization probes. Hybridization to and washing of filters were done according to instructions from the Biotrans protocol. Plaques that gave a positive hybridization signal were further purified and re-screened until single, well isolated plaques could be identified.

Each phage stock was titered and subjected to an in vivo excision process designed to remove cloned insert and pBluescript plasmid vector from the λ-ZAP phage vector following the Stratagene protocol. Plasmid inserts from plaque-purified clones were

excised in the presence of helper phage M13K07 to form packaged pBluescript (SK^-) containing the cloned sequence. To infect cells and induce plasmid formation, supernatant containing pBluescript plasmids, packaged in M13 phage particles, was collected and an aliquot was combined with XL1-Blue cells. The final selection was based upon ampicillin resistance after transformation.

Recombinant plasmids were isolated from the cultures by a rapid small scale DNA isolation procedure and digested with the restriction enzyme EcoRI to excise the inserts which were then subjected to electrophoresis. DNA was transferred from the gel onto a Biotrans nylon membrane and hybridized to the met-6^+ probe. The largest EcoRI inserts were grown for large scale DNA isolation and these clones were sequenced as described in section 2.7.

2.9.2 5'-Rapid Amplification of cDNA Ends

The 5'-RACE (Rapid Amplification of cDNA Ends; Frohman et al., 1988) techniques were performed using a 5'-RACE kit (Bethesda Research Laboratories) following manufacturer's instructions. Total RNA from wild-type strain 74A was prepared as described in section 2.8. 10 µg of the RNA population were reverse transcribed in the presense of 25 pmol of gene-specific antisense primer to create the cDNA (-) strand, after which the unused gene-specific primer was removed by binding the cDNA strand to glass in the presence of sodium iodide (Vogelstein and Gillespie, 1979). Upon completion of the tailing reactions, aliquots of the amplification mixture were used for PCR. (dG)-adaptor primer (10 pmol), and nested gene-specific primer (25 pmol) was employed for the first round of amplification. Universal adaptor primer (25 pmol) and a nested amplification primer (25 pmol) were used to amplify second and third rounds of amplification.

2.10 Identification of Folate Polyglutamates

2.10.1 Folate Extraction

In studies of endogenous folate, appropriately supplemented Vogel's media (3L) were inoculated with conidia and grown with aeration at 30°C for 24 hours. For studies of folate synthesis, cultures (100 ml) received [^{14}C]p-ABA (10 µCi, 6.8 µmol/ml) supplements prior to growth as above. Mycelia for both types of experiment were harvested by vacuum filtration and the pads were washed with sterile ddH_2O before folate extraction.

Mycelial samples for extraction (≈ 10 g fresh weight for endogenous folates; ≈ 5 g for labeled folates) were finely cut and heated at 100°C for 10 min in 20 mM KH_2PO_4; 50 mM 2-mercaptoethanol; pH 7.0. After cooling and hand grinding with ≈ 2 g acid washed sand, the extract was again heated to 100°C for 10 min, cooled on ice and centrifuged at 15,000 x g for 10 min. Folates in the supernatant fractions were stored at -20°C for further analysis.

2.10.2 Folate Cleavage and HPLC Analysis

Folylpolyglutamates from mycelial extracts were quantitatively cleaved to p-$ABAGlu_n$ and pterin derivatives by zinc cleavage under acid conditions (Shane, 1986). The resulting p-$ABAGlu_n$ were converted to azo dyes of naphthylethylenediamine and were separated from the pterin derivative by chromatography on BioGel P2. The purified azo dyes were then reductively reconverted to p-$ABAGlu_n$ by treatment with Zn/HCl treatment (Shane, 1986), and the solutions were concentrated to dryness at 50°C under vacuum. The resulting residues were redissolved in sterile ddH_2O, adjusted to pH 6.5, centrifuged, and passed through 0.45 µm syringe filters.

Aliquots of p-$ABAGlu_n$ were subjected to HPLC analysis following the method of Shane (1986). The p-$ABAGlu_n$ samples were separated by HPLC using a Varian 5000

chromatography system and a 4.6 x 250 mm (ID) column of Partisil 10 SAX (Whatman) with 200 µl injection volume. Polyglutamate samples, derived from endogenous mycelial extracts, were assayed for p-ABAGlu$_n$ by measuring peak absorbance at 280 nm. Quantitative determinations were based on calibration curves that related peak area counts to nmol p-ABAGlu$_n$ injected (Imeson et al., 1990) to determine the distribution of naturally occurring polyglutamate derivatives. Polyglutamate samples, derived from ring labeled [^{14}C]p-ABA, were detected in 1 ml fractions of the column effluent. [^{14}C]p-ABAGlu$_n$ were detected by scintillation counting .

2.11 Fractionation of Cytosolic FPGS Activities

2.11.1 Initial Purification of FPGS Protein

To minimize losses, all steps in the purification procedure were carried out at 4°C and all buffers contained the protease inhibitors benzamidine (1 mM) and PMSF (1 mM), added fresh to buffers prior to use. Mycelial pads for each strain (≈ 40 g fresh weight for molecular weight determination, ≈100 g fresh weight for wild-type FPGS isolation) were harvested, washed in ddH_2O and homogenized in a blender (1:1.6 w/v) in extraction buffer (20 mM potassium phosphate, 50 mM ß-mercaptoethanol, 20% glycerol, 1 mM PMSF and 1 mM benzamidine, pH 7.4; designated buffer A). Each homogenate was then passed through a continuous flow sonicator (Branson Sonic Cell Disruptor 350; at least 5 passes) and clarified by centrifugation at 4,500 x g for 10 minutes to give a crude extract, designated Step 1 protein.

Streptomycin sulphate (10% w/v; dissolved in buffer A, pH 7.4) was added to the crude extract (Step 1 protein) to give a final concentration of 1% (Cichowicz and Shane, 1987a), and the solution was stirred for 1 hour. The precipitate was removed by centrifugation at 6500 x g for 10 minutes using a JA-20 rotor (Beckman). The supernatant (Step 2 protein) was then fractioned using ammonium sulphate as decribed by Chan et al.

(1991). After 1 hour of stirring, the first precipitate (0-45% of saturation) was removed by centrifugation at 10,000 x g for 10 minutes and discarded. Further addition of ammonium sulphate were made to increase the supernatant to 70% of saturation. The solution was stirred for 1 hour and the precipitate was collected by centrifugation at 10,000 x g for 10 minutes. The resulting pellet was resuspended in buffer A to give Step 3 protein.

2.11.2 Apparent Molecular Weight Determination of FPGS

Step 3 protein pellets were redissolved in 5-15 ml buffer A without glycerol. The extract of each strain was applied to a 2.6 x 80 cm column of Sephadex G-200 (Pharmacia LKB Biotechnology Inc.) pre-equilibrated with buffer A without glycerol. Protein was eluted at a flow rate of 0.5 ml/min, and fractions of 3 ml were collected. 200 µl of alternate fractions were assayed for FPGS activity (see section 2.11). Determinations of apparent molecular weight were based on the elution volumes (Andrews, 1964) of FPGS-active peaks from a column calibrated with the following marker proteins: ß-amylase 200 kDa; alcohol dehydrogenase 150 kDa; bovine serum albumin 66 kDa; carbonic anhydrase 29 kDa; and cytochrome c 12.4 kDa. Blue dextran was used to determine the void volume of the column.

2.11.3 Isolation and Purification of Wild-Type FPGS

Pellets of wild-type Lindegren A Step 3 protein were redissolved in a small volume (5-25 ml) of glycerol containing buffer A and desalted by passage through 1.6 x 12 cm columns of BioGel P6-DG pre-equilibrated with buffer A. The desalted Step 3 protein was immediately applied to a 5 x 5 cm column of DE-52 cellulose pre-equilibrated with buffer B (20 mM potassium phosphate, 10 mM KCl, 50 mM 2-mercaptoethanol, 20% glycerol, 1 mM PMSF, 1 mM benzamidine, pH 7.0). After addition of the sample, the column was washed with 300 ml of the above buffer. FPGS activity was eluted by applying a 600 ml linear KCl gradient (300 ml of buffer B containing 10 mM KCl in the

mixing vessel to 300 ml of the same buffer in the reservoir but containing 600 mM KCl). 10 ml fractions were collected and FPGS-active fractions were pooled (Step 4 protein) and desalted with BioGel P6-DG columns.

Further purification of FPGS protein was achieved by applying desalted Step 4 protein to a 2.5 x 10 cm Reactive Green 5 column pre-equilibrated with buffer A. After loading FPGS protein, the column was washed with 100 ml buffer A, followed by elution with a 200 ml linear NaCl gradient (0 to 0.5 M NaCl in extraction buffer). 10 ml fractions were collected and those with FPGS activity were pooled to give Step 5 protein.

After concentration by ultrafiltration with an Amicon stirred cell and desalting through BioGel P6-DG columns, the resulting Step 5 protein was applied to a 2.5 x 10 cm column of Heparin agarose pre-equilibrated with buffer A, pH 7.0. The column was washed with 100 ml of extraction buffer, followed by elution with a 200 ml linear NaCl gradient (0 to 0.5 M NaCl in buffer A) collected in 10 ml fractions. The pooled FPGS-active fractions (Step 6 protein) were desalted through BioGel P6-DG columns equilibrated and washed with buffer C (10 mM potassium phosphate, 50 mM ß-mercaptoethanol, 20% glycerol,1 mM benzamidine, 1 mM PMSF, pH 7.4). The resulting desalted Step 6 protein was applied to a 2.5 x 5 cm column of hydroxylapatite pre-equilibrated with the 10 mM potassium phosphate buffer as above. FPGS was not retained under these conditions and was collected in the column wash as Step 7 protein. The entire column wash was diluted two-fold with buffer A without glycerol and was applied to a MonoQ-HR5/5 FPLC column pre-equilibrated with buffer A containing 10% v/v glycerol. A KCl gradient (10 to 600 mM) in this buffer was used, fractions of 2 ml were collected and those containing FPGS activity were pooled to give Step 8 protein.

The apparent molecular weight of wild-type FPGS was also determined by comparison of electrophoretic mobility of purified protein with that of known molecular weight standards following SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out on 4% stacking and 12% running discontinuous SDS-PAGE mini slab gels

according to the method of Laemmli (1970). Protein samples were denatured by the addition of 4 volumes of denaturation buffer (0.0625 M Tris-HCl, 10% glycerol, 0.02% SDS, 0.05% ß-mercaptoethanol, 0.00125% bromophenol blue) and boiling for 5 minutes. Proteins were stained with either BioRad Coomassie blue or silver stain kits as per manufacturer's instructions.

2.12 Assay of FPGS Activity

2.12.1 Preparation of Folate Substrate

[6R,S]tetrahydrofolates ($H_4PteGlu_n$, n=1,2) were generated by catalytic conversion of pteroylglutamate or pteroyldiglutamate with platinum oxide and hydrogen gas (Reid and Friedkin, 1973). Conversion was checked by measuring increased absorbance of the solution at 296 nm (Blakley, 1969). When conversion was completed, concentrated 2-mercaptoethanol was added to give a final concentration of 1.0 M, and platinum oxide was removed by centrifugation. The solution was stored at -20°C in the dark.

2.12.2 Measurement of FPGS Activity

The FPGS assay was based on the incorporation of [^{3}H]L-glutamate into reduced folate to form labeled folylpolyglutamate. The assay, modified after Pristupa et al. (1984), was a 1.15 ml standard reaction system containing Tris-HCl pH 10.3, 200 µmol; KCl, 30 µmol; $MgCl_2$, 10 µmol; HCHO, 1 µmol; ATP, 5 µmol; [R,S]H_4PteGlu, 0.1 µmol; 2-mercaptoethanol, 100 µmol; L-glutamate, 1.5 µmol containing 2.5 µCi of [^{3}H]L-glutamate; and 200 µl extract protein. The reaction tubes were purged with H_2 and incubated at 37°C for 2 hr. The reaction was terminated by adding ice-cold 30 mM 2-mercaptoethanol and incubating at 100°C for 5 min. Boiled reaction mixtures were centrifuged in a table top centrifuge to remove precipitated protein (if necessary), and the tubes were then stored at 4°C prior to isolation of the labeled product.

[^{3}H]folylpolyglutamate products were isolated by ion exchange chromatography on

DE-52 cellulose, a modification of the technique of McGuire et al. (1980). Reaction assays were applied to 1 x 3 cm columns of Whatman DE-52 cellulose (fines removed) equilibrated with wash buffer (80 mM NaCl, 10 mM Tris-HCl, pH 7.5). A 0.5 cm layer of acid-washed sea sand was placed on top of the bed to prevent cellulose disturbance. A 1 ml rinse of each reaction tube with wash buffer was also added to the columns after the sample had penetrated the beds. Absorbed folate product was washed with 30 x 1 ml aliquots of wash buffer to remove unincorporated [^{3}H]glutamate. Elution of labeled polyglutamates was accomplished by washing each column with 6 x 1 ml of 0.1 N HCl, collected as one 6 ml fraction. 1 ml aliquots of this acid fraction was assessed for radioactivity by liquid scintillation counting (see section 2.13). Control assays lacking either tetrahydrofolate or enzyme were run for each sample to allow correction for any non-folate dependent incorporation of [^{3}H] into the polyglutamate fraction.

2.13 Measurement of Radioactivity by Liquid Scintillation

All radioactive samples were counted in a Tracor Analytic Liquid Scintillation counting system (Mark III, Model 6881), a Beckman LS7500, or a Beckman LS6000TA liquid scintillation counter. In experiments involving [^{3}H] and [^{14}C], Bray's scintillation fluor (Bray, 1960) was used to measure radioactivity (prepared from scintillation grade chemicals supplied by Sigma Chemical Co. and Fisher Scientific). In experiments involving [^{35}S], Amersham aqueous scintillation fluor was used.

2.14 Protein Determination

Protein samples containing high levels of 2-mercaptoethanol were assayed using the method of Bradford (1976). Bovine serum albumin (Calbiochem or Sigma, Fraction V) was used to prepare standard curves for each assay.

3. Results

The relationship between met-6 and its probable alleles met-s2706 and mac are not clear. Genetic studies support the possibility of one gene, but do not exclude the possibility that met-6 and mac are closely-linked, but non-allelic, loci (Murray, 1969). Studies of the met-6 and mac mutants revealed that the biosynthesis of folylpolyglutamates by N. crassa involves at least two activities (Ritari et al, 1973a,b, Cossins and Chan, 1983, 1984) and suggested that two enzymes could be responsible for production of the polyglutamate found in this species. This thesis was a further investigation of the relationship between the met-6 and mac mutations.

3.1 Transformation of Polyglutamate-Deficient Mutants to Identify the Met-6$^+$ Gene

In order to study the nature of the two FPGS activities (equations 4a and 4b), the gene encoding the putative FPGS protein (the met-6$^+$ gene) was identified and cloned by complementation of the methionine-auxotrophic mutants met-6, met-s2706 and mac to restore wild-type phenotype. Characterization involved transforming mutant cell lines with CsCl-purified cosmid DNA vectors 4:11:C, 7:10:D and pSV50-4.1.3.1, each containing a wild-type met-6$^+$ gene and a benomyl-resistant gene for selection. Cosmids 4:11:C and 7:10:D were gifts from Dr. T. Schmidhauser. The cosmid pSV50-4.1.3.1 was isolated from a Neurospora crassa genomic cosmid library of strain 74A DNA from our laboratory (Drygas et al., 1989).

The met-6$^+$ cosmids were tested for their ability to transform each of the three mutants and stable methionine prototrophic, benomyl resistant colonies were obtained, providing evidence for the stable insertion of DNA. The number of transformant colonies observed ranged from 50 - 250 per µg input DNA when starting with 5 x 10^6 spheroplast cells. The same spheroplasts were transformed with CsCl-purified pSV50 vector as a

negative control, and the number of transformants observed were ≤ 1 per µg input pSV50 DNA.

Subcloning the met-6$^+$ gene from the cosmid pSV50-4.1.3.1 was necessary for the characterization of the gene by DNA sequence analysis, since the cosmid was approximately 40 kb in size. The strategy to determine the portion of the cosmid containing the met-6$^+$ gene, and the construction of plasmid pIA50 containing the met-6$^+$ gene, is described in Figure 3. Met-6 mutant spheroplasts were cotransformed with cosmid pSV50 and various restriction endonuclease fragments generated from the pSV50-4.1.3.1 cosmid (Figure 3). Transformation results indicated that the enzymes EcoRI, PstI, and HindIII do not interrupt the met-6$^+$ gene since methionine prototrophic, benomyl resistant transformants were observed when spheroplasts were cotransformed with cosmid pSV50 and EcoRI, PstI, and HindIII-cut fragments (Table 8). However, the restriction enzymes KpnI, BglII and SalI did interrupt the met-6$^+$ gene, as transformation frequencies using fragments generated with these enzymes approximated that of the negative pSV50 control (Table 8).

To determine which digests would be most useful for subcloning the met-6$^+$ gene, the electrophoretic patterns of pSV50-4.1.3.1 separately digested with the restriction endonucleases EcoRI and PstI were analyzed. Isolation of approximately 5 µg of individual restriction endonuclease-cut fragments, followed by cotransformation with pSV50 of met-6 mutant spheroplasts, showed that a 5 kb EcoRI fragment and a 6 kb PstI fragment were capable of producing the methionine prototrophic and benomyl resistant transformants from met-6 spheroplasts. The frequencies of positive transformation were ≥ 85% of that seen in the positive EcoRI-cut cosmid control transformation. Upon further analysis using restriction enzymes in combination, it was revealed that a 4.5 kb EcoRI/PstI fragment was capable of giving positive transformation. This fragment was subcloned into plasmid vector pUC19 to generate plasmid pIA23 (Figure 3), which was able to complement met-6 mutant spheroplasts.

FIGURE 3

CONSTRUCTION OF PLASMID pIA50 FROM COSMID pSV50-4.1.3.1 FOR THE STUDY OF NEUROSPORA FPGS

The EcoR1/BamH1 insert of pSV50-4.1.3.1 was subcloned into the plasmid pUC19 to produce pIA50. Further to produce plasmid pIA62 and pIA68. Details are given in the Results.

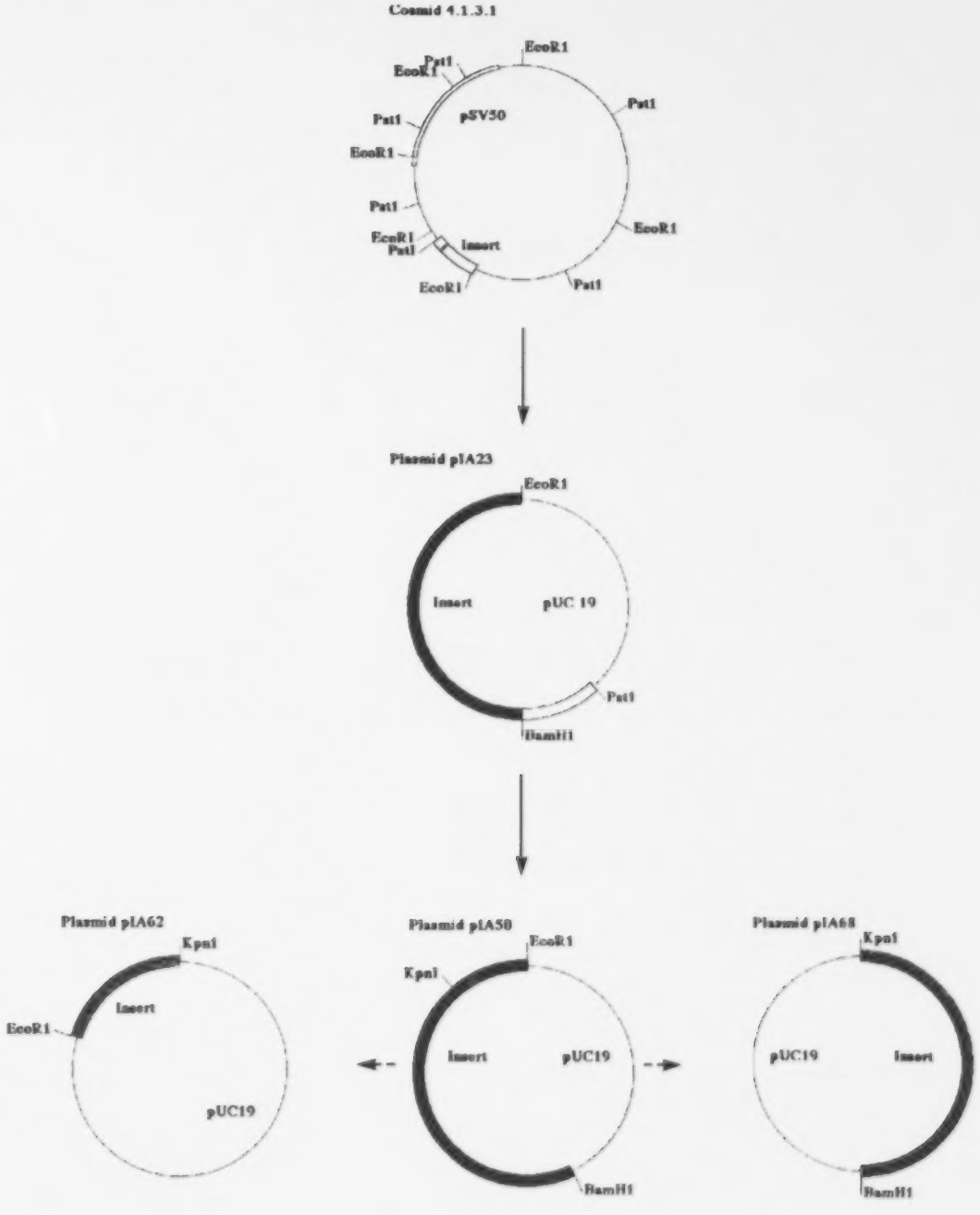

Cosmid 4.1.3.1
EcoR1
Pst1
EcoR1
Pst1
pSV50
Pst1
EcoR1
Pst1
EcoR1
Pst1
EcoR1
Insert
EcoR1
Pst1
Plasmid pIA23
EcoR1
Insert
pUC 19
Pst1
BamH1
Plasmid pIA62
Kpn1
Insert
EcoR1
pUC19
Plasmid pIA58
EcoR1
Kpn1
Insert
pUC19
BamH1
Plasmid pIA68
Kpn1
pUC19
Insert
BamH1

Table 8. Results of Transformation of Met-6 Spheroplasts with Digested pSV50-4.1.3.1

Restriction Enzyme	Transformation Result
BamH1	+
BglII	-
EcoR1	+
HindIII	+
Kpn1	-
Pst1	+
Sal1	-

+ = > 50 transformants per transformation

- = ≤ 10 transformants per transformation

In further subcloning of this fragment, the transforming activity was found to be contained on a 3.5 kb EcoRI/BamHI fragment located within the larger EcoRI/PstI fragment (Figure 3) of plasmid pIA23. This 3.5 kb EcoRI/BamHI fragment was subcloned into pUC19 to generate plasmid pIA50, which was able to rescue all three mutants to prototrophy. The entire insert of plasmid pIA50 was sequenced as seen in section 3.3 and was used for further analysis of the met-6$^+$ gene as described in subsequent sections.

The functional region of plasmid pIA50 which contained the putative met-6$^+$ gene was confirmed by cotransformation of the met-6 mutant with the cosmid pSV50 plus linear restriction fragments generated from plasmid pIA50. Results were similar to those from digestion of the larger cosmid pSV50-4.1.3.1 source: control EcoRI-cut pIA50 + pSV50 was able to positively transform the met-6 mutant spheroplasts, while KpnI-cut and SalI-cut pIA50 plus cosmid pSV50 lacked the ability to transform the mutant met-6 cells to prototrophy. KpnI digestion of pIA50 produced a single 6.2 kb band; KpnI digestion of the 3.5 kb EcoRI/BamHI fragment produced two fragments of 0.7 kb and 2.7 kb respectively. The EcoRI/KpnI fragment (0.7 kb) was cloned into pUC19 to form plasmid pIA62; the KpnI/BamHI fragment (2.7 kb) was cloned into pUC19 to form plasmid pIA68. Neither of the latter plasmids plus pSV50 were able to transform met-6 mutant spheroplasts to prototrophy.

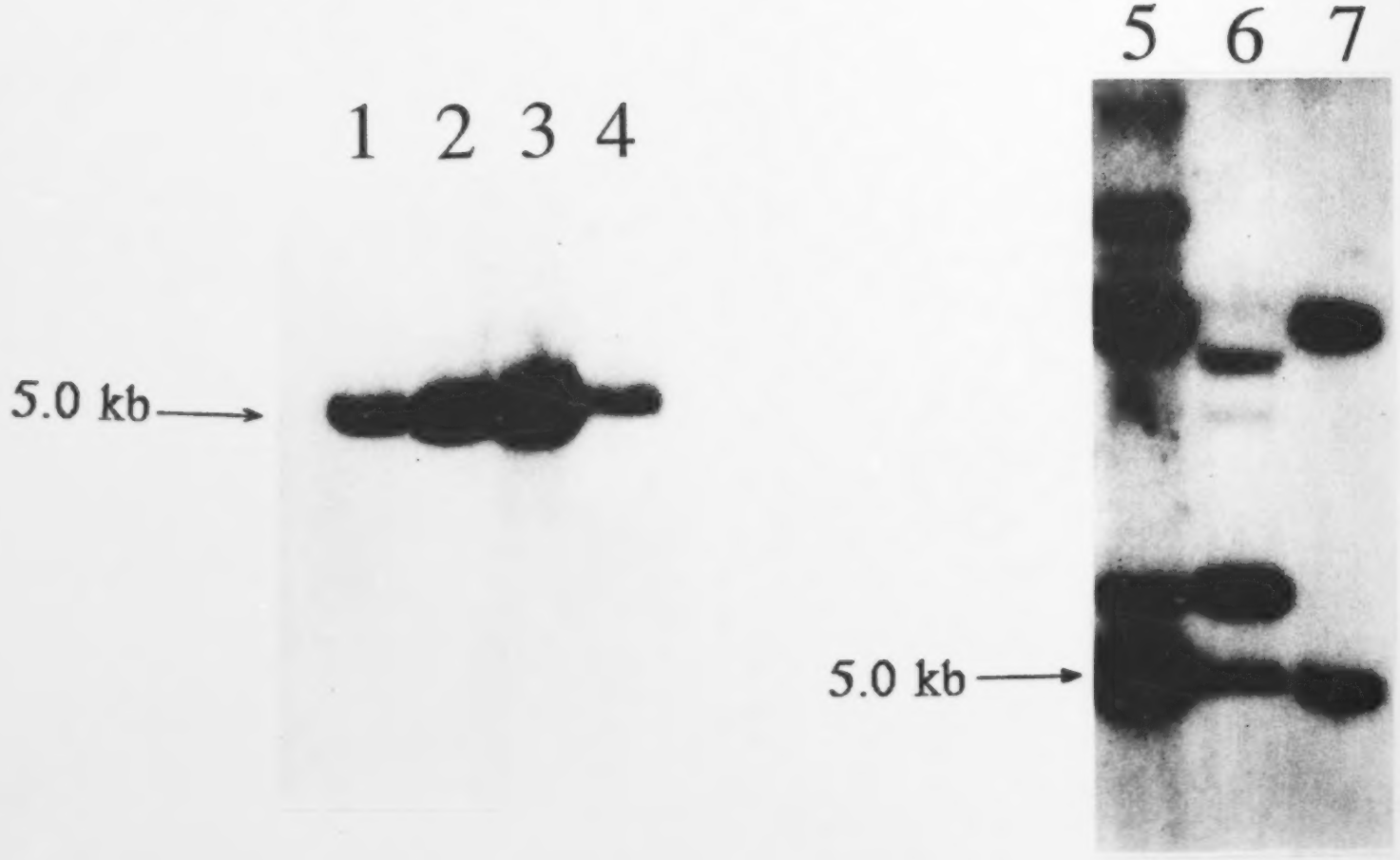
3.2 Southern Analysis of the Met-6$^+$ Locus in Genomic DNA

For Southern analysis of the putative met-6$^+$ gene, genomic DNAs were digested with restriction enzymes, electrophoresed, and blotted onto nylon membrane. The membrane was hybridized with [^{32}P]dCTP-labeled probe specific to the 2.7 kb fragment of pIA68 (see section 3.1; KpnI digestion). Genomic DNA isolated from the 74A wild-type and mutant strains were processed in parallel. When cut with EcoRI, one band of approximately 5.0 kb was observed in all lanes (Figure 4, lanes 1 to 4). This result

FIGURE 4

SOUTHERN BLOT ANALYSES OF N. CRASSA GENOMIC DNA.

The N. crassa DNAs were digested with EcoR1 and probed with a ^{32}P-labeled Kpn1/BamH1 gene fragment from pIA68. Wild type 74A, lane 1; met-6 mutant, lane 2; mac mutant, lane 3, met-s2706 mutant, lane 4; met-6 transformant, lane 5; mac transformant, lane 6, met-s2706 transformant, lane 7.


1 2 3 4
5.0 kb
5 6 7
5.0 kb

suggested that no gross deletions or insertions of the met-6$^+$ DNA were present in the mutant strains of Neurospora used in this study.

Transformations of N. crassa generally result in nonhomologous (often multiple) integration of transforming sequences (Akins and Lambowitz, 1985). The presence of successful ectopic insertion of the foreign plasmid DNA into mutant chromosomes is readily identified in Figure 4. The presence of two or more bands in lanes 5 to 7 indicated integration of one or more copies of the met-6$^+$ DNA present in the transformant strains. The rescuing bands appear large enough to accommodate a full met-6$^+$ gene.

3.3 Nucleotide and Deduced Amino Acid Sequence Analysis

DNA sequencing was performed on both strands of CsCl-purified plasmid pIA50 using synthetic 17 nucleotide long (17-mer) oligonucleotides that were complementary to the regions being sequenced. The nucleotide sequence of the entire 3.5 kb EcoRI/BamHI fragment of plasmid pIA50 is shown in Figure 5, indicating an insert of 3440 nucleotides. Computer analysis of the sequence was unable to reveal an uninterrupted open reading frame (ORF) long enough to encode a protein similar in size to other eukaryotic FPGS proteins, approximately 60 to 70 kD.

The possibility that a portion of the met-6$^+$ gene was present in a different reading frame was examined. After computer translations of all six potential reading frames were examined, two blocks of translated sequence showed significant homology to foreign FPGS genes within the 3.5 kb subclone . These two blocks of sequence were out of frame with each other and spanned a transition region between +126 and +192 that showed poor homology to foreign FPGS genes. As a result, this transition region was examined for intron/exon boundaries. A putative 60 nucleotide intron was identified at position +129 of the largest open reading frame, supporting the out of frame homology findings. Intron/exon boundaries at both the 5' and 3' ends of the putative intron showed strong

homology to Neurospora introns (see Section 3.5). This argues for the presence of an exon at the 5' end of the met-6$^+$ gene located out of frame with the 3'-terminus of the protein. The intron would be expected to bring the two sections with homology to the foreign FPGS genes back into frame.

After eliminating intron sequences detected using both cDNA analysis (see Section 3.5; panel 22-panel 23) and homology findings, above, (panel 24-panel 25), an ORF of 1587 nucleotides was identified which would give rise to a putative protein of 528 amino acids with a molecular weight of 58,982 Daltons. This ORF begins 471 nucleotides from the EcoRI site of plasmid pIA50 (Figure 5; panel 1). Based on size estimates of the wild-type FPGS protein (Chan et al., 1991; this study, Section 3.10), this 1587 nucleotide ORF could produce a protein similar in size to the wild-type FPGS protein. This product size was also in agreement with the FPGS proteins from other eukaryotic organisms which range between 60 and 70 kD. Translation initiation at a second in-frame AUG codon could also produce a protein of comparable size; a 1485 nucleotide ORF (with introns removed) would produce a protein containing 494 amino acids and having a molecular weight of 56,733 Daltons (Figure 5; panel 2).

Evaluation of the most appropriate translation start site was based on similarity to the N. crassa translational start consensus sequence (Bruchez et al., 1993; Legerton and Yanofsky, 1985).

Consensus sequence	(G)TCA(C)(C)ATGG A A A
Translation start site 1	G GCC T G ATGC
Translation start site 2	C GCA C C ATGG

Based on these considerations, site 2 would appear to be the most favourable choice. However, in the vast majority of cases in eukaryotes, including Neurospora, translation begins at the proximal methionine of the transcript (Kozak, 1983, 1989). Absolute confirmation of the translation start site remains to be achieved.

FIGURE 5

SEQUENCE OF PLASMID pIA50 AND SUBSEQUENT SEQUENCE

1. Translation Start #1. This is postulated to be the hypothetical start to the largest open reading frame of the met-6$^+$ gene based on sequence data.

2. Translation Start #2. This is postulated to be a second hypothetical start site, 102 nucleotides smaller than the largest open reading frame of the met-6$^+$ gene.

3. At position +63 of the largest open reading frame, the triplet codon CT**C** of pIA50, giving rise to leucine, is a CT**A** triplet codon in Lindegren A, Emerson, met-6, mac, and met-s2706, also giving rise to leucine.

4. At position +94 of the largest open reading frame, the triplet codon **A**AA of pIA50, giving rise to lysine, is a **C**AA triplet codon in Lindegren A, Emerson, met-6, mac and met-s2706, giving rise to glutamine.

5. At position +126 of the largest open reading frame, the triplet codon TA**C** of pIA50, giving rise to tyrosine, is a TA**T** triplet codon in Lindegren A, Emerson, met-6, mac and met-s2706, also giving rise to tyrosine.

6. At positions +9 within the 1st hypothetical intron of the largest open reading frame, the triplet codon CA**A** of pIA50, giving rise to a glutamine, is a CA**G** triplet codon in Lindegren A, Emerson, met-6, mac and met-s2706, also giving rise to a glutamine.

7. At positions +14 within the 1st hypothetical intron of the largest open reading frame, the triplet codon A**C**C of pIA50, giving rise to threonine, is an A**T**C codon in Lindegren A, Emerson, met-6, mac and met-s2706, giving rise to isoleucine.

8. At positions +18 within the 1st hypothetical intron of the largest open reading frame, the triplet codon TT**T** of pIA50 is a TT**C** codon in Lindegren A, Emerson, met-6, mac and met-s2706.

Figure 5 CON'T

9 & 10. At positions +19 and +20 within the 1st hypothetical intron of the largest open reading frame, the triplet codon **TAC** of pIA50 is a **CCC** codon in Lindegren A, Emerson, met-6, mac and met-s2706.

11. At position +319 of the largest open reading frame, the triplet codon **G**GC of pIA50, giving rise to glycine, is a **A**GC triplet codon in Lindegren A, Emerson, met-6, mac, and met-s2706, giving rise to serine.

12. At position +323 of the largest open reading frame, the triplet codon AT**C** of pIA50, giving rise to isoleucine, is a AT**T** triplet codon in Lindegren A, Emerson, met-6, mac, and met-s2706, giving rise to serine.

13. At position +372 of the largest open reading frame, the triplet codon GG**T** of pIA50, giving rise to glycine, is a GG**C** triplet codon in Lindegren A, Emerson, met-6, mac, and met-s2706, also giving rise to glycine.

14. At position +378 of the largest open reading frame, the triplet codon CC**T** of pIA50, giving rise to a proline, is a CC**C** triplet codon in Lindegren A, Emerson, met-6, mac, and met-s2706, also giving rise to a proline.

15. At position +412 of the largest open reading frame, the triplet codon CT**C** of pIA50 and Emerson, giving rise to leucine, is a CT**A** triplet codon in Lindegren A, met-6, mac, and met-s2706, also giving rise to leucine.

16. At position +415 of the largest open reading frame, the triplet codon AT**T** of pIA50 and Emerson, giving rise to isoleucine, is a AT**C** triplet codon in Lindegren A, met-6, mac, and met-s2706, also giving rise to isoleucine.

17. At position +510 of the largest open reading frame, the triplet codon TC**T** of pIA50, Lindegren A, met-6, mac, and met-s2706, giving rise to serine, is a TC**C** triplet codon in Emerson, also giving rise to serine.

Figure 5 CON'T

18. At position +677 of the largest open reading frame, the triplet codon GTC of pIA50, met-6, mac, and met-s2706 giving rise to valine, is a GCC triplet codon in Lindegren A and Emerson, giving rise to alanine.

19. At position +401 of the largest open reading frame, the triplet codon TCC of pIA50, Lindegren A, Emerson, mac, and met-s2706, giving rise to serine, is a CCC triplet codon in met-6 mutant, giving rise to proline. This is the site of the met-6 mutation.

20. pcD21 5' end. This occurs at position +184 of the largest open reading frame and is postulated to be truncated and not to be the true 5' end of the cDNA.

21. pcD21 3' end. This occurs at position +1807 of the largest open reading frame and is the end of the cDNA version of the transcript, followed by a 20 nucleotide poly(A) tail.

22. 2nd Intron 5' end. Based on data from pcD21, this is the beginning point of the 51 nucleotide intron at position +1333 of the largest open reading frame.

23. 2nd Intron 3' end. Based on data from pcD21, this is the end point of the 51 nucleotide intron.

24. Hypothetical 1st Intron 5' end. Based on a change of reading frames, intron/exon boundaries, and homology to foreign FPGS genes, this is the beginning point of a 60 nucleotide intron at position +129 of the largest open reading frame

25. Hypothetical 1st Intron 3' end. Based as above, this is the end point of the 51 nucleotide intron.

Underlined Areas - Refer to the Two Primers Used to Amplify the Wild-type and Mutant PCR Products of 2.2kb. For Further Information, Refer to Figure 8.

```
GAATTCAAGT GGAACCTGGC GCCTTAGACC GCTCGGCCAC CTTGCCATCG ATATTATATT TTTATCGTGA AAGTGGCTTT      80
TGAGGACATT AGGGAAGATG CTCAGCTTAA CTAGTAGCCA CAGCATAGAC TACCGGTAGT ACATAGCTGA GGTGGGGCC     160
AGGTCGTTTTT GGCGGGGTTC AAACACAGTT GAAAAAGCCA AGATCGGGTA TGTTACTGAC GTCGGTGTTC CAGAGCCGGC     240
GTGTCTAGAA TAGAGTTCTA ACCGAATGGG TAAGCCAAGC ATCGCGAACG GAACATCCCG TCTTCTTTTC TTTTACCATC     320
GAAGTGACTG TGAGTGACAA GGTGGGTTAG GATTGAATCA CAGCTGATGG GATTGGGAGT TTTGGTATTG TGAATGCCAC     400
TAACACCAGG GCAGCTATCA GACGCCTCGA GATACTACCC GATTATCACC TCTTTGAAAC CAACGGCCTG                 470
1                                                                                3
ATG CAC CAT GTT TTG AGG CCC ATA GCT TTT CGA CTT GCC CTC GTC TCC CCT CTG AGG TCG CTC ACC ATC ACC CAT CAT CAC  551
Met His His Val Leu Arg Pro Ile Ala Phe Arg Leu Ala Leu Val Ser Pro Leu Arg Ser Leu Thr Ile Thr His His His
                4           2                          5                   6       7   8 910
CTC TTC TTC ACA AAA CGC ACC ATG GCA TCC AGC GCT AGA ACA TAC AAT -GTG AGT CAA CCA ACC TTT TAC CGC TCC ACA GAA  632
Leu Phe Phe Thr Lys Arg Thr Met Ala Ser Ser Ala Arg Thr Tyr Asn val ser gln pro thr phe tyr arg ser thr glu

CCC CAA ACA CTC ACT CAC CAT ATC GTA G-GAC GCA ATC GAT GCG CTC AAC TCC CTC CAG ACC CCC TTC GCC GTC ATC GAA  710
pro gln thr leu thr his his ile val  Asp Ala Ile Asp Ala Leu Asn Ser Leu Gln Thr Pro Phe Ala Val Ile Glu
20
GCC CGG CGC AAA GCG GGT ATC CGT CCC GAT GCG CAC TCG GTA AAG GAA ATG CGT GCC TAC CTC GCC CGC ATC GGC TAC  788
Ala Arg Arg Lys Ala Gly Ile Arg Pro Asp Ala His Ser Val Lys Glu Met Arg Ala Tyr Leu Ala Arg Ile Gly Tyr
                                                                          11  12
TCC TCC CAA GAT CTC GAT CGC CTC AAC ATC GTC CAC GTA GCC GGT ACC AAG GGC AAG GGC GGC ACC TGT GCC TTT GTC GAC  869
Ser Ser Gln Asp Leu Asp Arg Leu Asn Ile Val His Val Ala Gly Thr Lys Gly Lys Gly Gly Thr Cys Ala Phe Val Asp
                                  13      14                        19                15  16
TCC ATC CTC ACG CGT CAT CAG CGG ACT CAC GGT ATC CCT AGG CGC ATC GGT CTC TTC ACC TCC CCC CAT CTC ATT GCT GTC  950
Ser Ile Leu Thr Arg His Gln Arg Thr His Gly Ile Pro Arg Arg Ile Gly Leu Phe Thr Ser Pro His Leu Ile Ala Val

CGG GAA CGC ATC CGT ATC GAC TCC AAG CCC ATC TCC GAG GAA CTG TTT GCC CGC TAC TTC TTC GAG GTC TGG GAT CGT CTC 1031
Arg Glu Arg Ile Arg Ile Asp Ser Lys Pro Ile Ser Glu Glu Leu Phe Ala Arg Tyr Phe Phe Glu Val Trp Asp Arg Leu
      17
GAG ACA TCT CAG CTG GCT AAG GAT GAG GTG GAA CTG GGA AGC AAG CCC ATT TAC GCT CGC TAT CTT ACC TTG ATG AGT TAC 1112
Glu Thr Ser Gln Leu Ala Lys Asp Glu Val Glu Leu Gly Ser Lys Pro Ile Tyr Thr Arg Tyr Leu Thr Leu Met Ser Tyr

CAC GTC TAC CTG TCC GAG GGC GTC GAT GTA GCC ATT TAC GAA ACT GGC ATC GGG GGT GAA TAC GAT GCC ACC AAC GTA GTC 1193
His Ala Asn Leu Ser Glu Gly Val Asp Val Ala Ile Tyr Glu Thr Gly Ile Gly Gly Glu Tyr Asp Ala Thr Asn Val Val
                  18
GAC CGT CCC GTG GTC AGC GGT ATC AGC ACC CTT GGT ATC GAC CAC GTC TTT GTC CTG GGT GAT ACG GTC GAC AAG ATT GCG 1274
Asp Asn Pro Val Val Ser Gly Ile Ser Thr Leu Gly Ile Asp His Val Phe Val Leu Gly Asp Thr Val Asp Lys Ile Ala

TGG CAC AAG GCG GGT ATC ATG AAG ACG GGC AGC CCT GCG TTC ACG ATT GAG CAG GTT CCC TCC GCA ACT CAG GTG CTC AAG 1355
Trp His Lys Ala Gly Ile Met Lys Thr Gly Ser Pro Ala Phe Thr Ile Glu Gln Val Pro Ser Ala Thr Gln Val Leu Lys

GAT AGG GCG GTG GAA AAG GGC GTG GAT CTC AAG ATC CCT GAT GTT GAT CCC AGG CTC AAC GGC GTC AAG ATC CGT CCC GAT 1436
Asp Arg Ala Val Glu Lys Gly Val Asp Leu Lys Ile Leu Asp Val Asp Pro Arg Leu Asn Gly Val Lys Ile Arg Pro Asp
```

GCA GTC TTC CAG AAG AAG AAC GCA ACG CTG GCT ATC GCG CTC GCT GAG ACT GCC CTC AAA AAG TTG GAC CCT TCT TTC AAG 1517
Ala Val Phe Gln Lys Lys Asn Ala Thr Leu Ala Ile Ala Leu Ala Glu Thr Ala Leu Lys Lys Leu Asp Pro Ser Phe Lys

CCT GGC ACC GAC AGT CTA TCA CCC GAG TTT GTC CAA GGC CTC GAA CAG GTT GTC TGG CGT GGC CGA TGC GAG GTC AAG GAG 1598
Pro Gly Thr Asp Ser Leu Ser Pro Glu Phe Val Gln Gly Leu Glu Gln Val Val Trp Arg Gly Arg Cys Glu Val Lys Glu

GAG GAT CAG GCT GTC TGG CAT CTT GAT GGC GCA CAT ACC GTT GAC AGC CTG AAG GTG GCG GGG AGA TGG TTC GTC GAG GAG 1679
Glu Asp Gln Ala Val Trp His Leu Asp Gly Ala His Thr Val Asp Ser Leu Lys Val Ala Gly Arg Trp Phe Val Glu Glu

TGT GTC AAG AAG GCC AAG GGC GGA CCC AAG GTG CTC ATC TTC AAC CAG CAA GGC CGG TCT GAG GCC GTT GAC TTC CTT GAT 1760
Cys Val Lys Lys Ala Lys Gly Gly Pro Lys Val Leu Ile Phe Asn Gln Gln Gly Arg Ser Glu Ala Val Asp Phe Leu Asp

GGG CTT TGC AAC ACT GTC AAG AGT GCT GAT CCG GAG GGA ACT GGC TTT AGC CAT GTG ATT TTT TGC ACC AAT GTG ACA TAT 1841
Gly Leu Cys Asn Thr Val Lys Ser Ala Asp Pro Glu Gly Thr Gly Phe Ser His Val Ile Phe Cys Thr Asn Val Thr Tyr
22 23
GCG ACG ACT GGC TAC AAA AAA G-GT ATG TTG TCC TGG GTC TTT CCA TGA TTT GCT CAA GCT AAC CTT CAA TAG-AC TTT GTC 1922
Ala Thr Thr Gly Tyr Lys Lys gly met leu ser trp val phe pro stop phe ala gln ala asn leu gln stop Asp Phe Val

AAC CAT CAA TAT AAC CCC AAG GAC ATT GAA AAC ATG ACA CAA CAA CGA GTC TTC GCG GAG AGG TGG TCG ACC CTC GAC CCC 2003
Asn His Gln Tyr Asn Pro Lys Asp Ile Glu Asn Met Thr Gln Gln Arg Val Phe Ala Glu Arg Trp Ser Thr Leu Asp Pro

TCT GCG AAT GTC ATG CTT ATC CCC ACT ATT GAG GAG GCC ATC AAC AAG GCC AGG AGC CTG GTA GAC ACC ACG GAA GGC GAA 2084
Ser Ala Asn Val Met Leu Ile Pro Thr Ile Glu Glu Ala Ile Asn Lys Ala Arg Ser Leu Val Asp Thr Thr Glu Gly Glu

CAG AAA GTG CAG GCA CTC ATC ACC GGC AGC CTT CAC CTT GTG GGT GGC GCG CTC GGC ATT TTG GAA AAA GCA GAT GCT CTC 2165
Gln Lys Val Gln Ala Leu Ile Thr Gly Ser Leu His Leu Val Gly Gly Ser Leu Gly Ile Leu Glu Lys Ala Asp Ala Leu

TGA A GGGGACTTGT GGTTCGGCAG CAGTTTTCAA CATTTATATT TTAGATATCC GTCCATTTTT TACATGGTAT 2239
GTACAGGATT TGGCATTTTG CTTCGCAGCA TCTTTTCTTT GTTACAAAAC ACAGGGAAAT ACAGGAAAAG TACAGGAAAA 2319
TAGAACAAGG GGGTTAAAGG AAAACCGGCA AGCCAGGACT TAATATTCAA CATACTCAAC TGTCTTAGAA CGGACCCAAG 2399
21
AAGGTGGAAA GCTTCCCGTT ACAAGGACAT TACACGTCTT GCCGTCCTTC GCGCGCCTGC TCAGGATCTT CTGAACCTGT 2479
TCCATGGCCG GCTCTACTGC GTTGTACGAC GAGGTTCCGT CTCGCACTAG CTTCCTGAAT AATCTTCTGC CCCTGCAACT 2559
GCACCGCGAG ATCTCTCTCC GGCTCACCCT CTACCGGCGC CTTTTCCTCG TTCCTTGTGA GCACCGCGTA GGTGAACACC 2639
GGTGTCGTTG TCCGCTTTGC TTCTGCGCGG CCGCAAGAAG AGCCCTCAAC AAGACTCCGG GTCCCTATCC TGCTGGTTGA 2719
ACACCAAGAC GTTGACATCG GCCTCCTTCA CCTTCCCAGA AAACCACTGT CCAACACCCG CCAGGCTATC CTCCGTATGC 2799
GCTCCATCAA CAAACACTCC GTGCCCTTGG GGTCTCTAAA AGACTCGCAC CTGCCCTTAA GGCTAGCCTC CTTCAAACCC 2879
TTCTCAAACT CAGCCGGATA TCATCCAATT TCCAGCCTTC CTCCCCAAAA GCCCCCGCGA ATTTGTGTCC CACCCTGGTA 2959
AGTGCTCCCT CGCCCCCCAC GCCAACGCCA TGTTAAACTT TTGAAATGGC CCTTCCAACT TGGCCCCCTC TACGCCCTTC 3039
CACTTCTCAA CCTGCTCATC CTCCACTTCT ACCAGCCTCT CCACTTCCTT CTCCTGCGCT CTATTCCTCA ACACCTCCAT 3119
CACTCCTTTC TGAGTTTCCC ATCCAGCCTC CTCGTAAACC CTTTGACCCT TCCTAAAATC CGCTCTTATT CCACGCAAAT 3199
TTTCTCAAGG GTATTTCCCA ACATAGCCAC GTGATCAATT CCCAGCTCGG TGACCACGCT CGCCGTCAGC ACTGCGGGCT 3279
CAAGAAGTTC GTCGAGTCGT ATTCCCCGCC AATCCCACAC TCAATCACCG CTTGCTTGAC CCCTCGCGAC AAAGATGTGA 3359
AAGGCCAGGA TGGTCAAGAA CCGAAAGTAA AACGGTTTCG TGCCGGGCCT GTCATACTCT GCCTCGGACT GAACCGGATC C 3440

The possibility that a portion of the FPGS gene might be present in 5' or 3' flanking regions outside the putative ORF was also examined. Computer translation of all six potential reading frames of the 3.5 kb subclone showed that only in regions within the putative ORF was there any significant homology to foreign FPGS genes. In addition, a 2.2 kb PCR fragment of wild-type genomic DNA generated from priming just outside the ORF was capable of restoring prototrophy to the mutant spheroplasts (see Section 3.6). This argued against part of the gene existing in the region outside the PCR product.

In an attempt to identify the preferred transcriptional start sequence, the genomic sequence was examined for motifs characteristic of eukaryotic promoter elements, such as TATA boxes (Neurospora consensus sequence = TATATAA) and CAAT boxes (Neurospora consensus sequence = GG(C/T)CAATCT) (Bruchez et al., 1993); computer analysis using PCGENE™ software showed no obvious similarities to such elements that might indicate a transcription start site upstream of the putative translation initiation site. However, many fungal genes lack such elements (Gurr et al., 1987). A CAAT-like sequence, GTTCAAACA, was observed 294 nucleotides upstream from the AUG start codon of the long ORF. In addition, two TATA-like sequences were detected at position -29 (ATTATCA) and -55 (TATCAGA) from the AUG start were identified.

In addition to these regulatory sequences, the putative ORF was examined for the presence of mitochondrial targeting sequences. About 20% of recoverable FPGS activity is associated with the mitochondrial fraction (McDonald et al., 1995), which precipitates with ammonium sulphate at 0-35% of saturation (Cossins and Chan, 1983). It has been shown that subcellular compartmentalization of FPGS is significant in Neurospora as evidenced by the effect that mutations have upon mitochondrial levels of this enzyme. The mutants met-6 and mac lack the mitochondrial FPGS activity in addition to the cytosolic FPGS function. A mitochondrial targeting sequence is most often found at the N-terminus of mitochondrial proteins (Hartl et al., 1989). Computer analysis using PCGENE™ software identified a putative targeting peptide, predicted in position +1 to +41. Interpretation of the ORF

sequence in this region revealed that it is rich in hydroxylated and basic amino acids and devoid of acidic amino acids, all characteristics of mitochondrial targeting peptides (Hartl et al., 1989).

Codon usage within the longest ORF (Table 9) was similar to that of other Neurospora genes (Kinnard and Fincham, 1983; Orbach et al., 1986). Where possible, C in the third position of the codon tends to be favoured (224 of 528 total codons), and there is a bias against A in the third position (only 56 of 528 total codons). Extreme bias is shown against A in the third position of highly expressed genes like am (1 codon ending in A of 454 total codons) or tub-2 (4 codons ending in A of 448 total codons). However, the putative met-6$^+$ gene did not show the extreme bias demonstrated by these highly expressed Neurospora genes, suggesting that it may not be highly expressed. This is in agreement with subsequent findings suggesting a low level of met-6$^+$ transcript (see Section 3.4) and of FPGS enzyme (see Section 3.10) in Neurospora cells.

The nucleotide-derived amino acid sequence of the largest ORF was compared to four known FPGS proteins (Figure 6). There was considerable similarity between this hypothetical met-6$^+$ gene product of Neurospora and the sequences searched. The similarity was concentrated in regions which corresponded to the major conserved regions between the four foreign FPGS proteins, primarily in the N-terminal regions (Figure 6). The overall percentage identity of the five proteins to the putative met-6$^+$ gene ranged from 20% (E. coli) to 43% (Yeast).

The 1587 bp Neurospora met-6$^+$ ORF contained regions with strong homology to the areas described as A and B regions of nucleotide binding sites for the E. coli folC gene product and the human FPGS sequence deduced from cDNA (Bognar et al., 1987; Toy and Bognar, 1990; Garrow et al., 1992). These sites occurred at amino acids 98-107 (A region) and 202-220 (inverted B region) of the putative met-6$^+$ gene product of the largest ORF, shown below (Figure 5; underlined).

Table 9. Codon Usage of the Largest Open Reading Frame of the Met-6$^+$ Gene

A. Met-6$^+$ Gene Codon Usage. Total Codons = 548

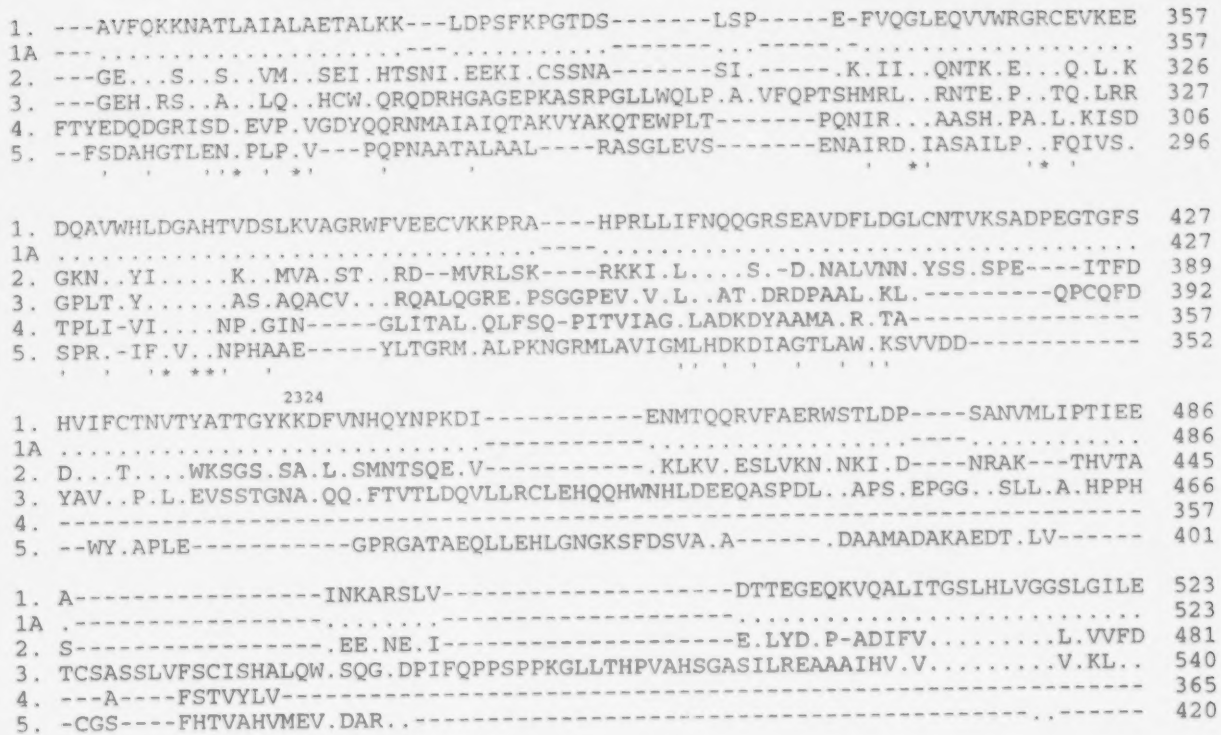
TTT	Phe	08	1.5%	TCT	Ser	04	0.7%	TAT	Tyr	03	0.5%	TGT	Cys	02	0.3%
TTC	Phe	13	2.4%	TCC	Ser	11	2.0%	TAC	Tyr	10	1.8%	TGC	Cys	03	0.5%
TTA	Leu	00	0.0%	TCA	Ser	01	0.1%	TAA	---	00	0.0%	TGA	Trp	00	0.0%
TTG	Leu	05	0.9%	TCG	Ser	03	0.5%	TAG	---	00	0.0%	TGG	Trp	06	1.1%
CTT	Leu	10	1.8%	CCT	Pro	05	0.9%	CAT	His	09	1.7%	CGT	Arg	08	1.5%
CTC	Leu	22	4.1%	CCC	Pro	14	2.6%	CAC	His	10	1.8%	CGC	Arg	08	1.5%
CTA	Leu	01	0.1%	CCA	Pro	02	0.3%	CAA	Gln	06	1.1%	CGA	Arg	03	0.5%
CTG	Leu	10	1.8%	CCG	Pro	01	0.1%	CAG	Gln	11	2.0%	CGG	Arg	04	0.7%
ATT	Ile	09	1.7%	ACT	Thr	08	1.5%	AAT	Asn	03	0.5%	AGT	Ser	03	0.7%
ATC	Ile	23	4.3%	ACC	Thr	16	3.0%	AAC	Asn	11	2.0%	AGC	Ser	09	1.7%
ATA	Ile	01	0.1%	ACA	Thr	05	0.9%	AAA	Lys	07	1.3%	AGA	Arg	02	0.3%
ATG	Met	07	1.3%	ACG	Thr	07	1.3%	AAG	Lys	24	4.5%	AGG	Arg	09	1.7%
GTT	Val	06	1.1%	GCT	Ala	10	1.8%	GAT	Asp	18	3.4%	GGT	Gly	10	1.8%
GTC	Val	25	4.7%	GCC	Ala	14	2.6%	GAC	Asp	15	2.8%	GGC	Gly	20	3.7%
GTA	Val	05	0.9%	GCA	Ala	08	1.5%	GAA	Glu	13	2.4%	GGA	Gly	02	0.3%
GTG	Val	10	1.8%	GCG	Ala	14	2.6%	GAG	Glu	18	3.4%	GGG	Gly	03	0.5%

FIGURE 6

COMPARISON OF THE AMINO ACID SEQUENCES OF EUKARYOTIC AND PROKARYOTIC FPGS PROTEINS.

The aligned sequences are Neurospora crassa (1. 74A wild-type; 1A. Emerson wild-type), Yeast FPGS (2. - Shane, personal communication), Human FPGS (3.- Garrow et al., 1992), Escherichia coli FPGS (4. - Bognar et al., 1987), and Lactobacillus casei FPGS (5. - Bognar et al., 1985). The alignment was made employing a Clustal matrix (PCGENE™). Periods (·) indicate identity with the 74A sequence at a site. Asterisks (*) indicate amino acid homology among the five proteins, a position that is perfectly conserved. A quotation mark (') indicates a position that is conserved in most cases. Underlined sequence indicates homology to the A and B nucleotide binding domains. Numbers above amino acids indicate landmarks seen in figure 5.

1
1. FPGS N.crassa 74A MHHVLRPIAFRLALVS 016
1A FPGS N.crassa Emerson 016
2. FPGS Yeast .---------- 001
3. FPGS Human .E---------- 002
4. FPGS L. casei .NYTET---------- 006
5. FPGS E. coli .IIKRT.Q.-----A. 010
3 4 2 52425 20
1. PLRSLTITHHHLFFTKRTMASSARTYNDAIDALNSLQTPFAVIEARRKAGIRPDAHSVKEMRAYLARIGYSSQD 090
1A .....Q..... 090
2. ----------VGK..R..VT.....SNY.N.M.I.QT.D.KNTMTLL..HEWSR.....AS. 054
3. ----------.Q..VRM..T...NAGYL.QVKRQRGD.QTQ-LEA.EL....S.LQVE. 050
4. ----------VAYI-HFSPRL.KTGDH.RILTLLH.LGNPQQQGR---------- 040
5. ..A.W----------LSY.EN.HSKTIDLGLE.VSLVAARL-G.LKPAPF---------- 050
1112 1314 19*1516
1. LDRLNIVHVAGTKGKGGTCAFVDSILTRHQRTHGIPRRIGLFTSPHLIAVRERIRIDSKPISEELFARYFFEVW 164
1A .....S..... 164
2. FNK.....IT.....S.A..TS...GQYK--EQL..-...YS....KS.....NGE.....K..K..... 125
3. .....I..T.....S....TEC..-----RSYGL-KT.F.....VQ.....NGQ...P...TK..WRLY 118
4. -----YI..T..N...SAANAIAHV.------EASGLTV..Y...FIMRFN...M..HE..PDAALVNAVAF.R 103
5. -----VFT....N...T..RTLE...------MAAGYKV.VYS....VRYT..V.VQGQELP.SAHTAS.A.I- 112
17 18
1. DRLE--TSQLAK-DEVELGSKPIYARYLTLMSYHVYLSEGVDVAIYETGIGGEYDATNVVDRPVVSGISTLGID 235
1A ....--.....-.....A..... 235
2. ...DST..S.D.FPHMIP....G.FKF...L.F.TFIQ.DCKSCV..V.V...L.S..IIEK.I.C.VTL.... 199
3. H...ET-------KDGSCV.M.P.F.F....AF..F.Q.K..L.VV.V....A..C..IIRK...C.V.S.... 185
4. AAL.RL-------QQQQADFNVTEFEFI.ALG.WYFRQRQ....VI.V....DT.S...IT-....VLTEVAL. 169
5. ------------ESARGDISLT.FE.G...ALWLFKQAQL..V.L.V.L..RL....I..-AD.AVVTSIAL. 172
1. HVFVLGDTVDKIAWH-KAGIMKTGSPAFTIE-QVPSATQVLKDRAVEKGVDLKILDVDPRLNG----VKIRPD- 302
1A .....-.....-.....----..... 302
2. .TSL....IEE...N-.G..F.S.A....V.K.P.QGLTI..E..E.RKTT.TEVPPFKQ.EN-----.LGIA- 267
3. .TSL.....E....Q-.G..F.Q.V....VL-.PEGPLA..R...QQISCP.YLCPMLEA.EEGGPPLTLGLE- 256
4. .QKL..H.ITA..K.-....I.R.I.VV.-GNL..D.AA.VAAKVATT.S--QW.RF.RDFSVPKAKLHGWGQR 239
5. .TDW..PDRES.GREA....FRSEK..IV-GE--.EMPSTIA.V.Q...AL.QRRG.EWNYSVTD---HDWA-- 238



RESIDUE SIZE

1. KADAL	FPGS	N.crassa	528	residues
1A				
2. RIDVK	FPGS	Yeast	486	residues
3. P.LSQ	FPGS	Human	545	residues
4. -----	FPGS	L.casei	365	residues
5. ----K	FPGS	E. coli	423	residues

	A Region	Inverted B Region
Consensus	V-X-G-T-X-G-K-G-(S)-T T	D-X_4-E-V-G-(I)-G-G-X-X-D-X-T-N-(I)(I) L V V
74A Genomic sequence	V-A-G-T-K-G-K-G- G -T	D-X_4-E-T-G--I--G-G-E-Y-D-A-T-N--V-V

3.4 Northern Analysis of the Met-6$^+$ Transcript

To examine transcription of the putative met-6$^+$ gene, poly(A) mRNA of 74A wild-type was isolated, electrophoresed, transferred to nylon membrane and probed with the 2.7 kb KpnI/BamHI fragment of pIA68 labeled with ^{32}P dCTP. The probe detected a single transcript band of 2.0 kb, the presumptive met-6$^+$ message (Figure 7, A, lane 1), after isolation of total RNA from an 8 hour culture. Using poly(A) RNA from mycelia from 24 hour old cultures, the probe was unable to detect a signal (Figure 7, A, lane 2). The time-course for met-6$^+$ mRNA in cultures was further examined by probing blots of RNA isolated from 0, 6, 12, 18 and 24 hour cultures. Transcript levels gradually increased during the first 3 isolation times, peaking at the 12 hour culture (Figure 7, B).

3.5 Isolation of a Met-6$^+$ cDNA Clone

In order to determine the positions of introns as well as to identify the 5' and 3' ends of the mRNA transcript of the met-6$^+$ gene, met-6$^+$ cDNA clones were isolated and sequenced. A Neurospora cDNA library of strain 74A constructed in the λ–ZAP/EcoRI vector (Stratagene) was generously provided by Dr. Matthew Sachs (Stanford, California). Clones from the plated library were probed with a radiolabeled 2.7 kb KpnI/BamHI fragment of plasmid pIA68, and those producing a positive signal were purified and rescreened until single, well isolated plaques could be identified. Individual phage plaques were subjected to in vivo excision, followed by phagemid infection to form double-stranded plasmid (see Materials and Methods).

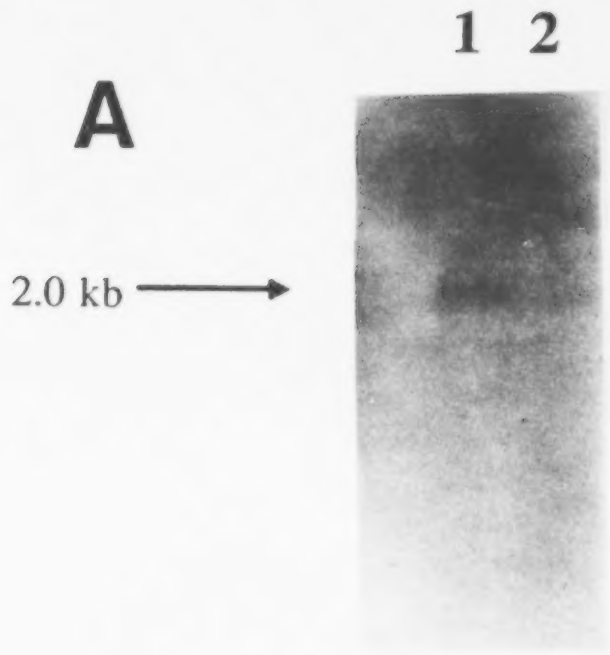
FIGURE 7

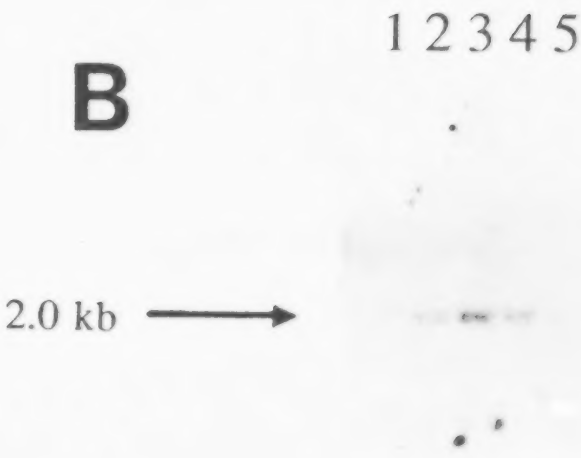
NORTHERN BLOT ANALYSES OF N. CRASSA mRNA.

Total or poly(A) mRNA was isolated from wild type 74A mycelia. The RNA was probed with a ^{32}P-labeled Kpn1/BamH1 fragment from pIA68 to detect met-6^{+} mRNA and with a ^{32}P labeled tub-2 ß-tubulin fragment to detect tub-2 mRNA.

A . 50 µg 74A wild type total RNA, 8 hour germination prior to harvest, lane 1; 10 µg 74A wild type poly(A) RNA, 24 hour germination prior to harvest, lane 2.

B. 50 µg 74A wild type total RNA, 0 hour germination prior to harvest, lane 1; , 6 hour germination prior to harvest, lane 2; 12 hour germination prior to harvest, lane 3; 18 hour germination prior to harvest, lane 4; 24 hour germination prior to harvest, lane 5.


1 2
A
2.0 kb


1 2 3 4 5
B
2.0 kb

Three cDNA clones with an estimated size of 1.6 kb were obtained which had poly(A) tails and sequence matching known sequence of pIA50 and genomic sequence of wild-type 74A. Sequence at the 3' end of each of the three cDNA clones revealed that the transcript ended 252 nucleotides past the TGA stop codon of the met-6$^+$ ORF sequence (Figure 5; 4), beyond which was a 20 nucleotide poly(A) tail. The 5' end of the three cDNA clones started 304 nucleotides downstream of the first putative initiator AUG codon of the largest ORF (Figure 5; 3), with each of the three clones having the same 5' end position. The remainder of the nucleotide sequence and deduced amino acid sequence of the entire pcD21 clone (one of the three cDNA clones) corresponded exactly to the met-6$^+$ sequence derived from genomic DNA (with the exception of a single intron; see below). These clones did not appear to be full length and seemed not to contain the 5' end of the cDNA. To confirm this hypothesis, cotransformations of met-6 mutant spheroplasts with pcD21 and pSV50 were unable to transform the mutant to prototrophy. Since full length cDNAs of other genes can restore function in the appropriate mutants, the lack of transformation potential is not simply due to the lack of promoter sequences on the cDNAs (F.E. Nargang, personal communication). Thus, it appears that the cDNAs isolated here do not contain the entire gene. These cDNAs are probably the result of premature termination of the reverse transcriptase reaction (Beale et al., 1985).

The DNA sequence of pcD21 demonstrated the presence of an intron at nucleotide 1333 of the ORF (Figure 5; 5 - 5' end of the intron; 6 - 3' end of the intron), a deletion of 51 nucleotides from the genomic sequence. The boundaries of the genomic sequence at the site of the intron appeared to have strong homology with the consensus sequence of Neurospora exon/intron boundaries, shown below.

Genomic sequence 5' G // GTA T GT----------T AG // A 3'

Consensus 5' GTA(A)GT----------(C)AG 3'
C T

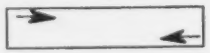
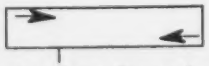
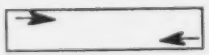
In a further attempt to identify the 5' end of the met-6 transcript, 5'-RACE was performed using a number of different approaches. Preliminary attempts at isolating the 5' region of the cDNA using RACE were not successful. In addition, no plaques hybridizing to met-6$^+$ probes could be deteced in a new cDNA library constructed in our laboratory.

3.6 Cloning and Sequencing of Mutant Genes

In order to identify the mutant sites of the met-6$^+$ gene in the met-6, met-s2706 and mac mutant strains, PCR-amplification of mutant genomic DNA was performed to isolate the three mutant forms of the met-6$^+$ gene. Standard PCR protocols were performed with 100 ng of mutant genomic DNA as described in the Materials and Methods, using Vent DNA Polymerase instead of Taq DNA Polymerase to reduce polymerase mismatch errors which would be mistaken for mutations. PCR products of 2.2 kb were generated for each mutant from oligonucleotide primers located outside the largest open reading frame (Figure 5, underlined). The PCR-amplified products were cloned into the vector pGEM-T, a specific plasmid used for subcloning PCR-generated fragments.

The cloned 2.2 kb PCR-generated fragment of the wild-type was able to rescue all three mutants to prototrophy (Figure 8). Not surprisingly, the individual 2.2 kb mutant genomic DNAs were unable to rescue homologous mutant spheroplasts (Figure 8). However, the cloned PCR-generated 2.2 kb genomic fragments derived from the three mutants were able to rescue each of the other mutants (Figure 8). The complete sequence of each of the 2.2 kb mutant clones was determined and compared with the met-6$^+$ gene sequence of the 74A wild-type strain found in plasmid pIA50. Many changes were detected, identified as changes numbered 3 through 18 in Figure 5 and Table 10. Because the genomic background on origin of the mutants is unclear, nucleotide characterization of additional wild-type strains were performed to determine if the changes were significant or merely genetic polymorphisms. Most of the changes were seen to be polymorphisms as

Figure 8. Transformation of Mutant Spheroplasts with Wild-type and Mutant PCR-generated Genomic DNA

Fragment	Description	Ability to Transform to Prototrophy*		
		met-6	mac	met-s2706
	genomic Eco-RI-BamHI (3.5 kb) fragment of 74A wild-type	+	+	+
	PCR product from 74A wild-type (2.2 kb)	+	+	+
 site of met-6 mutation	PCR product from met-6 (2.2 kb)	-	+	+
	PCR product from mac (2.2 kb)	+	-	-
	PCR product from met-s2706 (2.2 kb)	+	-	-
control	pSV50 cosmid	-	-	-

* + = > 50 transformants per transformation
- = ≤ 10 transformants per transformation

corresponding changes were present in one of the additional wild-type strains. Most of the changes were present in the wobble position of a codon and, as such, did not change the amino acid composition. In cases where there were inferred changes in amino acids between 74A wild-type and mutants, similar changes were evident between 74A and Lindegren A and/or Emerson wild-type strains (Figure 5). As a result, most of the changes encountered were not identified as mutations. The Lindegren A sequence was found to be closer in identity than the other wild-type sequences to the mutant sequences (Figure 5, Table 10). Although the backgrounds of the mutant strains were unclear, each appears very similar in sequence identity to the Lindegren A wild-type (Figure 5,Table 10).

The mutant PCR fragments were examined to identify any non-polymorphic changes in the nucleotide sequence to which the mutant phenotypes could be attributed. The met-6 mutant sequence was found to contain only one non-polymorphic change, a T to C nucleotide change at nucleotide position +400 of the open reading frame (Figure 5; Figure 9). The mutation resulted in a serine to proline change at amino acid 134 (Figures 5 and 6). This change was not present in any of the wild-type sequences, nor was it present in either the mac or met-s2706 mutant sequence. Preliminary sequences of the mac and met-s2706 mutants were examined and no non-polymorphic changes were detected. However, since cloned PCR fragments of each of the mutants were capable of restoring wild-type phenotype to the complementary mutant strains but not to themselves (Figure 8), it seems impossible that the mac and met-s2706 mutations are not present within the region sequenced. Further sequencing of the area especially in areas of compression using dITP sequence mixes should elucidate the sites of the remaining two mutations.

3.7 Identification of Endogenous Polyglutamate Pools by HPLC Analysis

Pteroylpolyglutamate synthesis in Neurospora has been primarily studied using older methodologies (Chan and Cossins, 1984). Although it is clear that Neurospora Lindegren A wild-type contains hexaglutamate derivatives of folate, precise identification of

Table 10. Polymorphisms Identified by Sequence Analysis.

Position Indicated on sequence (Fig. 5)	Strains					
	74A	Emerson	LinA	met-6	mac	met-s2706
3	CTC (Leu)	CTA (Leu)	CTA (Leu)	CTA (Leu)	CTA (Leu)	CTA (Leu)
4*	AAA (Lys)	CAA (Gln)	CAA (Gln)	CAA (Gln)	CAA (Gln)	CAA (Gln)
5	TAC (Tyr)	TAT (Tyr)	TAT (Tyr)	TAT (Tyr)	TAT (Tyr)	TAT (Tyr)
6	CAA (Gln)	CAG (Gln)	CAG (Gln)	CAG (Gln)	CAG (Gln)	CAG (Gln)
7*	ACC (Thr)	ATC (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)
8	TTT (Phe)	TTC (Phe)	TTC (Phe)	TTC (Phe)	TTC (Phe)	TTC (Phe)
9*	TAC (Tyr)	CCC (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)
10*	TAC (Tyr)	CCC (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)
11*	GGC (Gly)	AGC (Ser)	AGC (Ser)	AGC (Ser)	AGC (Ser)	AGC (Ser)
12*	ACC (Thr)	ATC (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)
13	GGT (Gly)	GGC (Gly)	GGC (Gly)	GGC (Gly)	GGC (Gly)	GGC (Gly)
14	CCT (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)	CCC (Pro)
15	CTC (Leu)	CTC (Leu)	CTA (Leu)	CTA (Leu)	CTA (Leu)	CTA (Leu)
16	ATT (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)	ATC (Ile)
17	TCT (Ser)	TCC (Ser)	TCT (Ser)	TCT (Ser)	TCT (Ser)	TCT (Ser)
18*	GTC (Val)	GCC (Ala)	GTC (Val)	GTC (Val)	GTC (Val)	GTC (Val)

Asterisks (*) indicate positions where base substitution has resulted in an amino acid change.

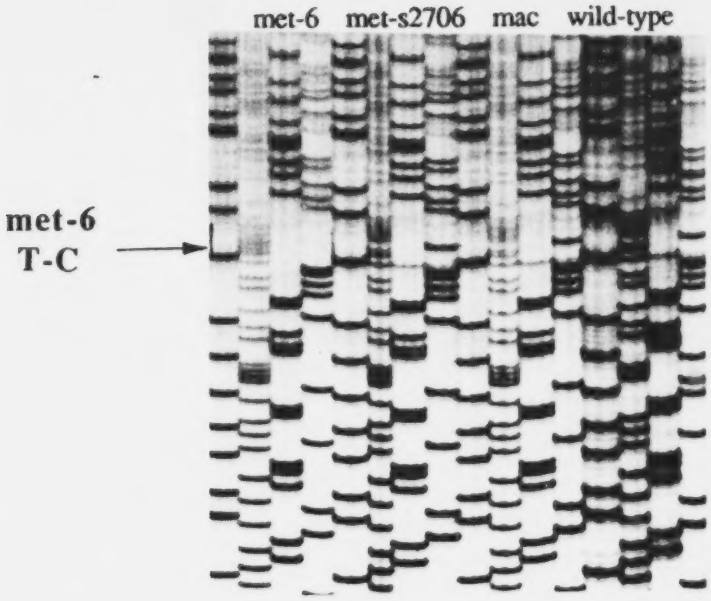
FIGURE 9

SEQUENCE ANALYSES OF N. CRASSA MET-6 MUTANT GENOMIC DNA.

The N. crassa DNAs were PCR amplified as per the Materials and Methods and sequenced.

A. Position 1 Met-6 mutant, lane 1-4, A,C,G and T lanes; Position 2 Met-s2706 mutant, lane 5-8, A,C,G and T lanes; Position 3 Mac mutant, lane 9-12, A,C,G and T lanes; Position 4 Wild-type 74A, lane 13-16, A,C,G and T lanes.

B. Position 1 Wild-type 74A, lane 1-4, A,C,G,T lanes; Position 2 Met-6 mutant, lane 5-8, A,C,G,T lanes.



Wild-type 74-A

Met-6

ACC **T**CC CCC

Thr **Ser** Pro

ACC **C**CC CCC

Thr **Pro** Pro

distinct polyglutamate peaks with a high level of sensitivity was limited to the resolving power of these techniques (Mullins and Duch, 1992). The endogenous folylpolyglutamate chain length distributions of the Lindegren A wild-type, met-6, mac and met-s2706 mutants, as well as cosmid transformed mutant strains were therefore studied using extracts of mycelia that were harvested 24 hr after spore inoculation. The folate polyglutamate pools were examined by reductive cleavage of folates, isolation of p-aminobenzoylpolyglutamates, and separation according to glutamyl chain length on HPLC. The quantitation of these derivatives was based on direct absorbance measurements at 280 nm (Shane, 1986), as described in Section 2.10.

The identification of each HPLC peak was based on a direct comparison of the retention times with those of authentic p-ABAGlu$_n$ standards. Figure 10 shows a typical separation of p-ABAGlu$_2$ to p-ABAGlu$_7$ standards that were generated by reductive cleavage of the corresponding PteGlu$_n$ derivatives (Shane, 1986). The HPLC conditions employed allowed good baseline separation of individual p-ABAGlu$_n$ derivatives, with retention times that were reproducible on repeated injections, and only affected by changing the buffer being used to elute the column. Slight differences in retention times were noted whenever a new batch of elution buffer was prepared.

A typical elution pattern of the p-ABA polyglutamates prepared from extracts of the Lindegren A wild-type are shown in Figure 11. The data in Table 11 suggest that the folates of Neurospora wild-type occurred almost exclusively as highly conjugated pteroylpolyglutamates with hexaglutamates accounting for the majority of the polyglutamate pool. Trace amounts of penta-, tetra- and triglutamate were also detected, but these represented a relatively small fraction (between 5% - 10%) of the total folate pool.

In contrast, highly conjugated polyglutamyl folates represented less than 10% of the polyglutamates detected in both met-6 and mac (Table 11). In these mutants, the conjugated folates were mainly short chain di- and triglutamates. Met-s2706, on the other

FIGURE 10

HPLC ELUTION PROFILE OF p-ABAGlu_{2-7} STANDARDS.

Individual folylpolyglutamate standards were produced from the corresponding $PteGlu_n$ derivatives by Zn-HCl treatment (see Materials and Methods). A 200 µl mixture of Glu_2 to Glu_7 was injected onto a 4.6 mm x 25 cm column of Whatman Partisil 10 SAX. Column temperature 35 °C, flow rate 1 ml/min, pH 6.5, UV detector 280 nm, recorder attenuation 64.

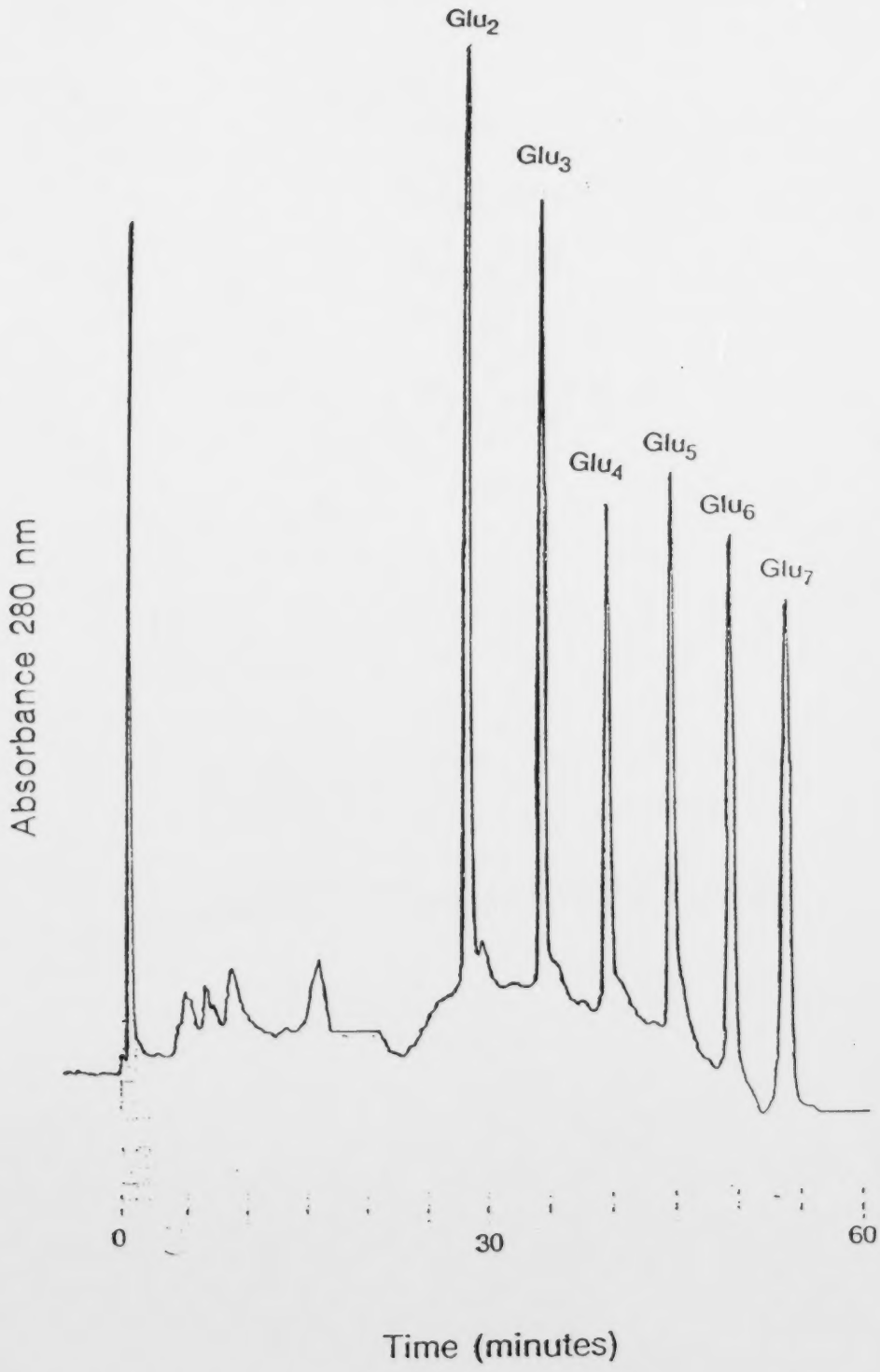

Glu2
Glu3
Glu4
Glu5
Glu6
Glu7
Absorbance 280 nm
0
30
60
Time (minutes)

FIGURE 11

HPLC CHROMATOGRAPHY OF NEUROSPORA WILD TYPE POLYGLUTAMATES.

Cultures of Lindegren A wild-type mycelia were extracted and the folates were cleaved to p-ABA polyglutamate derivatives (see Materials and Methods). 200 µl of the resulting polyglutamate extract (equivalent to approximately 4 g of fresh weight of tissue) were injected and subjected to HPLC analysis. Attenuation setting 16.

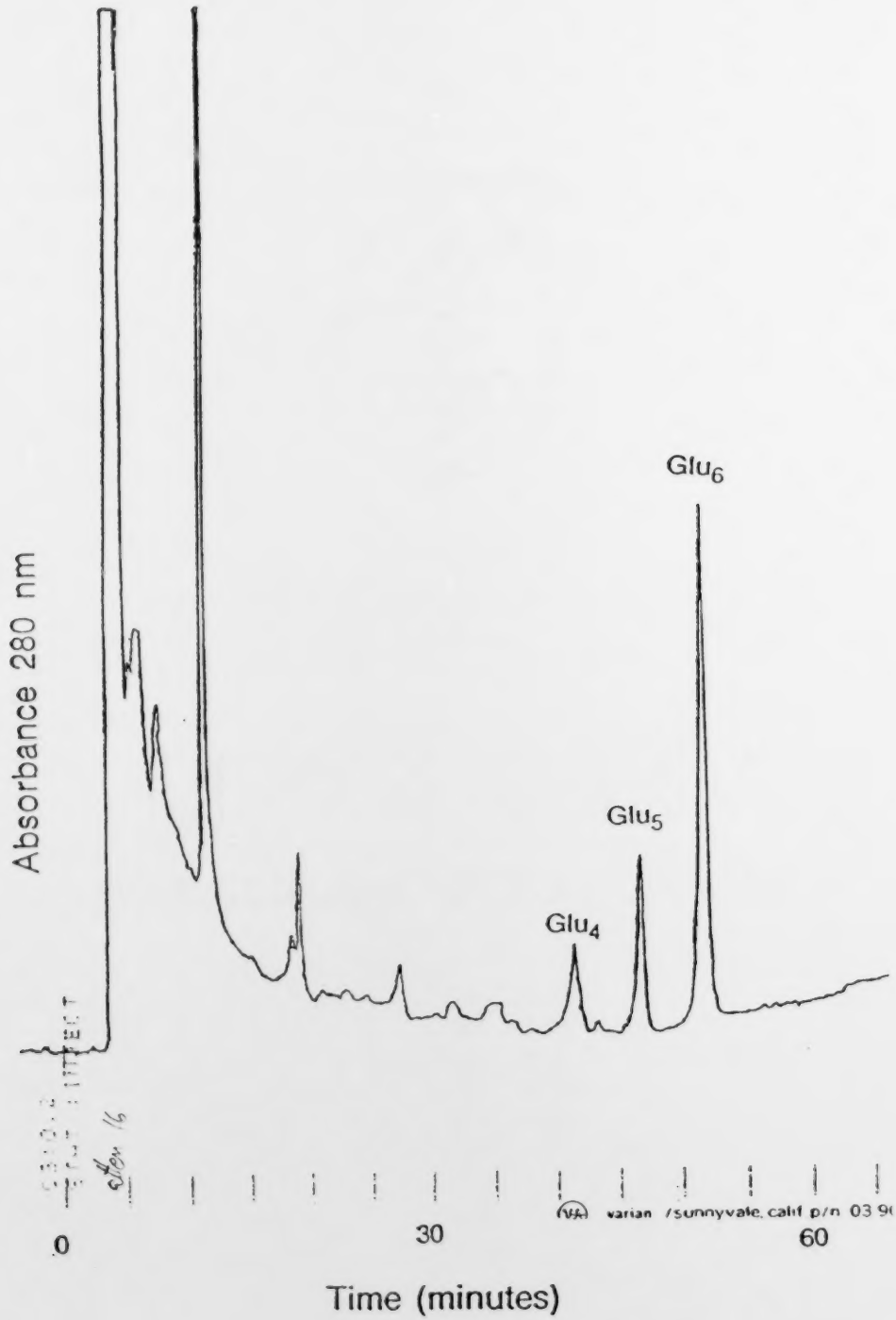

Absorbance 280 nm
Glu6
Glu5
Glu4
varian /sunnyvale, calif p/n 03 9
0
30
60
Time (minutes)

Table 11. Distribution of Intracellular Folates Isolated as p-ABA Polyglutamates from Various Strains of Neurospora crassa

Strain	Folates Recovered[a]	Polyglutamate Chain Length (%)				
		Glu_2	Glu_3	Glu_4	Glu_5	Glu_6
1. Lindegren A Wild-Type	60.20	n.d	n.d	0.7	2.0	97.3
2. Met-6 Mutant	52.54	71.1	13.8	11.6	2.6	0.8
3. Mac Mutant	44.28	84.7	6.0	2.5	3.6	3.2
4. Met-s2706 Mutant	37.26	n.d	n.d	1.0	6.6	92.4
5. Met-6 Transformant	71.56	7.2	7.5	11.6	13.7	60.1
6. Mac Transformant	100.09	3.3	2.6	8.7	13.9	71.6
7. Met-s2706 Transformant	75.51	0.5	7.5	7.5	10.0	74.5

a = Data are expressed as pmol p-ABAGlu_n (n=2 to 6) detected from extracts per g fresh weight. Note: the monoglutamate derivative is not clearly resolved from background absorbance.

n.d = not detected

hand, had observable conjugated folates that were almost exclusively hexaglutamates which was a similar distribution to the wild-type. The met-s2706 phenotype was also observed to be relatively leaky, as a result, significant growth was recorded in the absence of L-methionine supplements. This finding was in agreement with an earlier report (Cossins and Chan, 1985).

Transformation of met-6, met-s2706 and mac mutants with cosmid DNA containing the met-6$^+$ gene resulted in ability to generate long-chain polyglutamates in vivo. In each of the transformants examined, the hexaglutamate derivative was the major folate detected (Table 11). This distribution is similar to that associated with the wild-type, but distinct from that shown by extracts of met-6 and mac mutants. It is also clear from Table 11 that tetra- and pentaglutamates occurred in significant amounts in all of the transformant strains.

Because the mutants were grown under different media conditions than the wild-type or transformants, to obtain appropriate control conditions Lindegren A wild-type and transformant cultures were also grown in methionine-containing media. Analyses of polyglutamate distributions of these cultures showed similar distributions to cultures grown without methionine supplementation (data not shown).

3.8 Incorporation of [^{14}C]p-ABA into Folylpolyglutamates

Folate distributions were also examined after feeding ring-labeled [^{14}C]p-ABA. In these studies, the growth media of the Lindegren A wild-type, mutants, and cosmid transformed strains were supplemented with 10 μCi of ring-labeled [^{14}C]p-ABA (see Section 2.1), and the mycelial folates were extracted after a growth period of 24 hours. Previous work has shown that these conditions result in extensive labelling of the folate pools in N. crassa (Cossins and Chan, 1984). As before, folates were cleaved to p-ABAGlu$_n$ derivatives and recovered by HPLC as above (Shane, 1986), and 1 ml fractions were collected and assessed for radioactivity by scintillation counting. The incorporation of labeled p-ABA was evident in long-chained folates in the wild-type (Figure 12; Table

FIGURE 12

INCORPORATION OF ^{14}C p-ABA INTO NEUROSPORA WILD-TYPE FOLYLPOLYGLUTAMATES.

Conidia of the Lindegren A wild-type were germinated in the presence of a 10 μCi [^{14}C]p-ABA solution for 24 hours, folates were extracted, and then these folates were cleaved to p-ABA polyglutamate derivatives (see Materials and Methods). During HPLC analysis, the column effluent was collected in 1 ml fractions and assessed for radioactivity.

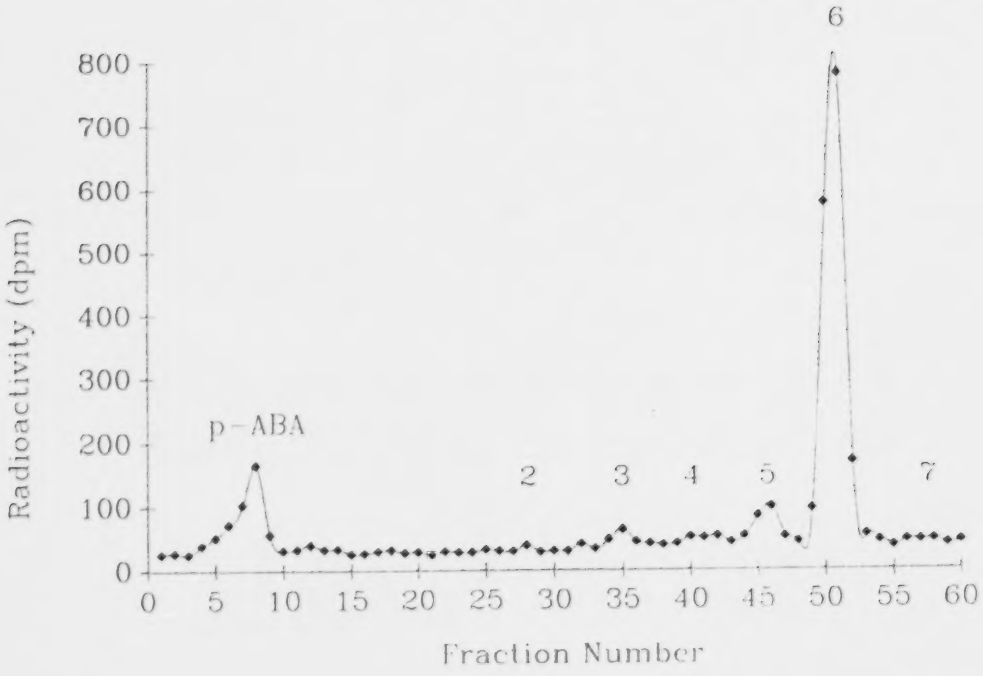

6
800
700
600
500
400
300
200
100
0
p-ABA
2
3
4
5
7
Radioactivity (dpm)
0 5 10 15 20 25 30 35 40 45 50 55 60
Fraction Number

Table 12. Distribution of Labeled Intracellular Folates Isolated as p-ABA Polyglutamates in Various Strains of Neurospora crassa.

Strain	Folates Recovered[a]	Polyglutamate Chain Length (%)					
		Glu_1	Glu_2	Glu_3	Glu_4	Glu_5	Glu_6
1. Lindegren A Wild-Type	1.95	2.7	2.2	4.6	4.0	12.4	74.0
2. Met-6 Mutant	0.10	0.8	49.2	22.0	18.6	10.2	n.d
3. Mac Mutant	0.14	91.3	8.8	n.d	n.d	n.d	n.d
4. Met-s2706 Mutant	1.30	n.d	4.6	8.3	10.2	16.5	60.3
5. Met-6 Transformant	1.13	0.3	n.d	4.0	5.5	7.3	82.9
6. Mac Transformant	1.58	4.0	0.1	3.8	7.3	9.8	62.5
7. Met-s2706 Transformant	1.54	n.d	n.d	14.5	20.1	8.3	57.0

a = Data are expressed as pmol [^{14}C]p-ABA incorporated per g fresh weight of mycelia.

n.d = not detected

Note: Glu_7 was detected in met-s2706 transformant but represented only 0.1 % of that recovered in total folates. Glu_7 was not detected in any of the other strains.

12), with hexaglutamates accounting for over 70% of the label detected after HPLC (see Section 3.10). Only minimal labelling was detected in shorter chain length derivatives.

In met-6, less [^{14}C] was incorporated into folate and these were derivatives of short chain length (Table 12). A similar situation occurred in the mac mutant (Table 12). Met-6 and mac mutants contained only trace amounts of radioactivity as hexaglutamate folate, the folates were mainly mono- and diglutamates. In contrast, the labeled folates of met-s2706 were mainly hexaglutamate derivatives. These data are in general agreement with that of Cossins and Chan (1985), who also reported that a relatively large percentage of the endogenous folate derivatives of met-s2706 was of higher glutamyl conjugation than the triglutamate.

Analyses of the transformants revealed polyglutamate distributions that were similar to those of the wild-type. Transformation and rescue of mutant strains deficient in polyglutamate synthesis established the generation of long-chain polyglutamates, with relatively large percentages of the supplied [^{14}C] being incorporated into the hexaglutamate derivative. Thus, transformation resulted in dominance of hexaglutamate as the major folate (Table 12). Also, significant amounts of label were associated with tetra- and pentaglutamates in these cultures.

3.9. Assay of FPGS Activities

To examine the FPGS protein of N. crassa mycelial extracts, FPGS activity was assayed by measuring the incorporation of tritiated glutamate into 5,10-methylene-$H_4PteGlu_n$ (n = 1 & 2). The assay used in this study was based on two previously published methods, the incorporation of [^{3}H]-labeled glutamate into polyglutamate derivatives (Pristupa et al., 1984; with modifications as described by Chan et al., 1991), and the removal of excess unincorporated [^{3}H]glutamate from labeled product by chromatography on DE-52 cellulose (McGuire et al., 1980).

As Neurospora FPGS products had not been previously separated by this method,

it was initially necessary to determine optimal conditions. Assay and elution conditions are shown in the Materials and Methods section. Figure 13 A summarize the elution patterns of tritiated glutamate as a control, Figure 13 B summarizes the elution patterns of [^{14}C]H_4PteGlu$_3$ as a control, and Figure 13 C summarizes a typical elution profile of an assay reaction mixture. These results show that complete separation of unincorporated [^{3}H]glutamate from radiolabeled folylpolyglutamates was achieved under the chosen conditions. These conditions were therefore used for the remainder of the present FPGS study.

3.10 Fractionation of Folylpolyglutamate Synthetase Activity and General Properties

3.10.1 Initial Purification

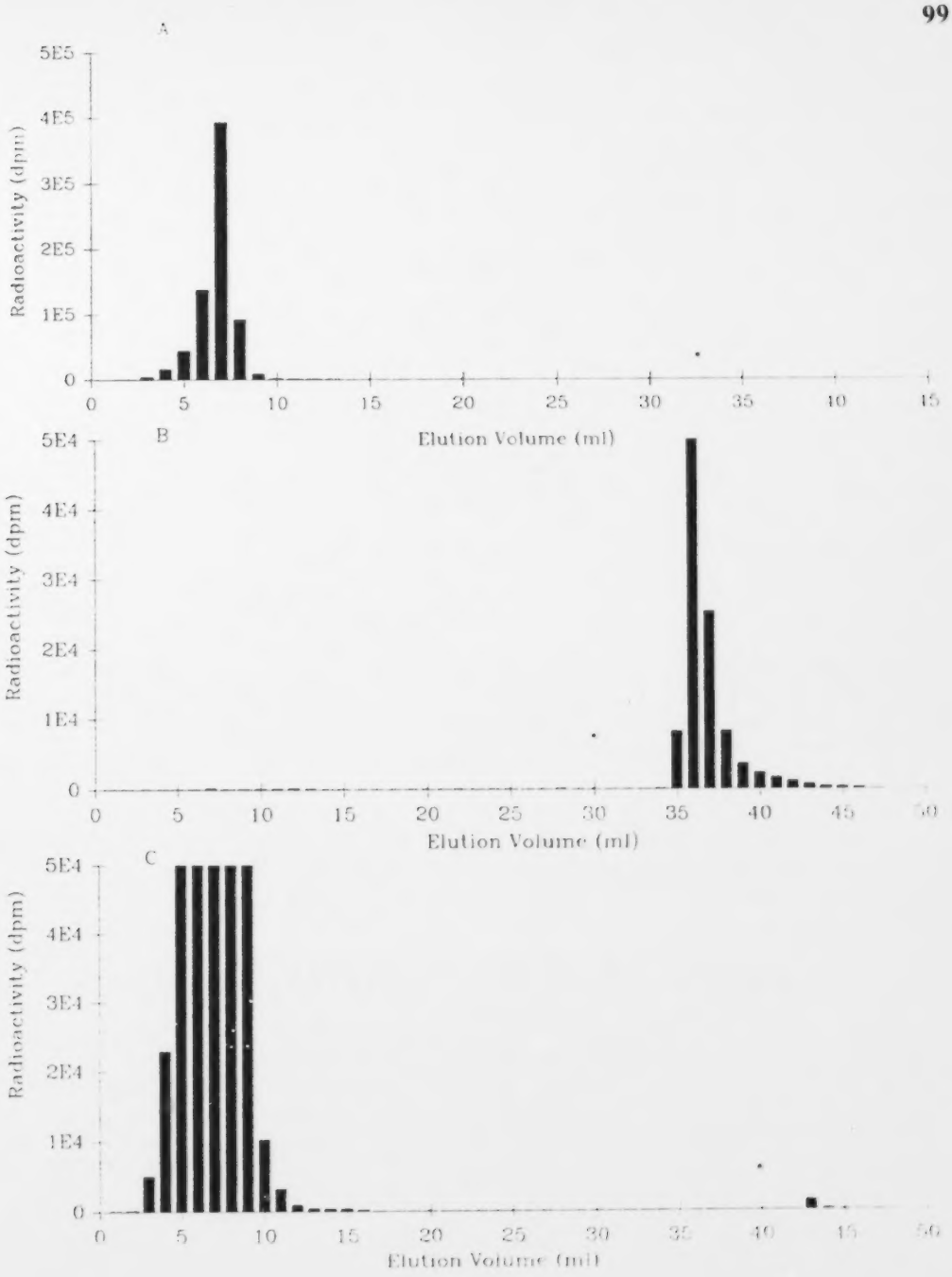
FPGS activity was partially purified and characterized using extracts of the wild-type, mutants, and transformants. Several workers have reported difficulty in purifying the folylpolyglutamate synthetases from bacterial, mammalian, and fungal systems, due to the extreme instability and low yield of these proteins (McGuire and Coward, 1984). Initial attempts to purify FPGS from 24 hr old Neurospora mycelia revealed very poor stability and large losses in activity at 4^{o}C (data not shown). As a result, buffers containing additional stabilizing agents, such as 20% glycerol and the protease inhibitors benzamidine and phenylmethylsulfonyl fluoride were employed in subsequent work to increase the final yields of isolated FPGS activity.

The first step in the purification involved homogenation/sonication of the cell suspension, followed by centrifugation to remove cellular debris (see Materials and Methods). The supernatant was adjusted to 1% with streptomycin sulphate to remove nucleic acids/polyanionic proteins, and to improve the fractionation of mycelial protein by

FIGURE 13

CHROMATOGRAPHIC SEPARATION OF [^{3}H]GLUTAMATE FROM [^{3}H]FOLYLPOLYGLUTAMATES.

Standard reaction mixtures (see Materials and Methods) were applied to 1 x 3 cm columns of DE52-cellulose equilibrated and washed with 10 mM Tris-HCl, 80 mM NaCl, pH 8.0 B, elution of unincorporated [^{3}H]glutamate. A, elution of [^{14}C]folyltriglutamate. C, elution of a wild type Step 3 protein reaction mixture. Column effluent was collected in 1 ml fractions and assessed for radioactivity. The arrows indicate the point at which 6 x 1 ml HCl was added.


A
5E5
4E5
3E5
2E5
1E5
0
Radioactivity (dpm)
0 5 10 15 20 25 30 35 40 45
B
5E4
4E4
3E4
2E4
1E4
0
Radioactivity (dpm)
0 5 10 15 20 25 30 35 40 45 50
Elution Volume (ml)
C
5E4
4E4
3E4
2E4
1E4
0
Radioactivity (dpm)
0 5 10 15 20 25 30 35 40 45 50
Elution Volume (ml)

ammonium sulphate precipitation (Step 3 - see Materials and Methods). Protein precipitating in the 45-70% range of saturation was resuspended in extraction buffer to form Step 3 protein, the common starting point of each of the two separate fractionation protocols seen in 3.10.1.1 and 3.10.1.2.

The ability of desalted Step 3 protein to incorporate [^{3}H]L-glutamate into 5,10-CH_2-H_4PteGlu$_n$ (n=1 & 2) is summarized in Table 13. In common with the wild-type, FPGS protein, isolated from the transformants, was able to utilize both folate substrates. On the other hand, FPGS protein of met-6 used only 5,10-CH_2-H_4PteGlu$_1$, whereas mac and met-s2706 used only 5,10-CH_2-H_4PteGlu$_2$ (Table 13), confirming previous work on the mutants (Cossins and Chan, 1984; Cossins and Chan, 1985). Although the chain lengths of the polyglutamates formed in these reaction systems were not examined it follows from earlier work (Cossins and Chan, 1984; Chan et al., 1991) that the wild-type FPGS protein would form 5,10-CH_2-H_4PteGlu$_n$ (n=3-5) from the diglutamate substrate under these assay conditions. It may also be assumed from the data in Table 13, that the three transformant strains converted the mono- and diglutamate substrate into polyglutamates with at least three glutamate residues. Thus the DNA insert used in these transformations produced expression of FPGS protein whose activities were complementary to that expressed by both mutant activities (equation 4a and 4b).

3.10.1.1 Molecular Weight Determination of FPGS

In further studies, Step 3 protein was redissolved in buffer A without glycerol and chromatographed on Sephadex G-200. This gel filtration resulted in an approximate 40 fold purification of FPGS activity for each strain, indicating that the specific enzyme activities were comparable (Table 14). When samples of Step 3 protein were chromatographed on Sephadex G-200 in the presence of marker proteins, it was clear that the wild-type and transformant enzymes had an apparent molecular weight of 66,000 $\pm$ 4,000 Da (Figure 14; Figure 15; Table 15). This value was in close agreement with earlier

Table 13. Folylpolyglutamate Synthetase Activity of 45-70% $(NH_4)_2SO_4$ Fractions.

Strain	Folate Substrate	
	CH_2-H_4PteGlu$_1$	CH_2-H_4PteGlu$_2$
1. Wild-Type	1.246	0.193
2. Met-6 Mutant	0.501	n.d.
3. Mac Mutant	n.d.	0.281
4. Met-s2706 Mutant	n.d.	0.223
5. Met-6 Transformant	0.938	0.198
6. Mac Transformant	0.706	0.146
7. Met-s2706 Transformant	1.106	0.232

Data are expressed as nmol of L-glutamate incorporated into 5,10-CH_2-H_4PteGlu$_n$ per mg protein.

n.d. = not detected

SDS-PAGE analyses of this protein from the wild-type, where a value of 65 kDa was noted (Chan et al., 1991). This value is also in close agreement with analyses of other eukaryotic FPGS proteins which range from 60 to 70 kDa (Shane, 1989), as well as estimates of the plasmid pIA50 open reading frame (section 3.3 - 58,982 Daltons). The gel filtration studies of wild-type, met-6 mutant and transformant FPGS proteins provided evidence for a single peak of FPGS activity that catalyzed the production of labeled polyglutamates (Figure 14). Similar elution profiles were seen with mac and met-s2706 mutants and transformants (Table 15). These gel filtration studies of wild type and transformant FPGS proteins also provided evidence for a single peak of FPGS activity that catalyzed the production of labeled polyglutamates from 5,10-methylenetetrahydrofolate and its diglutamate. Thus there was no direct support for two synthetase proteins of distinct molecular size being responsible for polyglutamate synthesis.

The apparent molecular weight values for FPGS protein isolated from the mutants also averaged near 66,000 as shown in Table 15. This implied that each mutation, although affecting the nature of the FPGS reaction, had little apparent effect on the overall size of the native protein. The transformant strains also showed only one peak of FPGS activity under these conditions. This observation provided additional evidence that only one size of FPGS protein was expressed by the strains examined.

3.10.1.2 Isolation and Purification of Wild-Type FPGS

To achieve further characterization of Neurospora FPGS protein, samples of Step 3 Lindegren A protein were redissolved in buffer A. After desalting by passage through 1.6 x 12 cm columns of BioGel P6-DG in buffer A, the desalted Step 3 protein was applied to a 5 x 5 cm column of DE-52 cellulose. After addition of the sample, the column was washed with 200 ml of buffer A to elute DHFS activity (McDonald et al., 1995), followed by a linear chloride gradient (200 ml buffer A to 200 ml buffer A containing 100 mM KCl) to recover FPGS protein. FPGS-active fractions were pooled and

Table 14. Fractionation of Neurospora Folylpolyglutamate Synthetase Activity.

Strain		Fractionation Step: Crude Extract	Streptomycin Sulphate	45-70% $(NH_4)_2SO_4$	Sephadex G-200
1. LinA	Activity	0.612	0.612	4.536	24.912
Wild-Type	Yield	100	99	181	76
2. Met-6	Activity	0.612	0.576	2.052	34.704
Mutant	Yield	100	109	132	75
3. Mac	Activity	0.252	0.252	0.972	12.348
Mutant	Yield	100	106	150	63
4. Met-s2706	Activity	0.383	0.383	1.442	15.504
Mutant	Yield	100	102	122	51
5. Met-6	Activity	0.756	0.720	3.564	12.960
Transformant	Yield	100	104	189	58
6. Mac	Activity	0.504	0.468	2.484	18.144
Transformant	Yield	100	101	207	74
7. Met-s2706	Activity	0.886	0.848	4.121	32.860
Transformant	Yield	100	98	134	61

Mycelia (ca. 40 g fresh weight) were harvested after 24 hours growth. Enzymatic activity is expressed in nmol L-glutamate incorporated into 5,10-CH_2-H_4PteGlu per hour at 37°C per mg protein. The substrate for the mac and met-s2706 mutant was 5,10-CH_2-H_4PteGlu$_2$. Yield is expressed as a percent. Increase in activity in Step 3 protein was associated with the removal of an FPGS inhibitor (Chan and Cossins, 1980).

Table 15. Determination of the Native Molecular Weight of Folylpolyglutamate Synthetases.

Strain	Ve/Vo	Molecular Weight (kDa)
1. Wild-Type	1.9326	66
2. Met-6 Mutant	1.9278	66
3. Mac Mutant	1.9407	65
4. Met-s2706 Mutant	1.8888	72
5. Met-6 Transformant	1.9214	67
6. Mac Transformant	1.9556	63
7. Met-s2706 Transformant	1.9545	63

Data are expressed as an average of three independent extractions

Data are calculated from the linear regresion log (Mr) = -0.8706 (Ve/Vo) + 3.5002, determined from molecular weight standards that were applied to the Sephadex G-200 gel filtration columns to produce the standard curve (Figure 14; Andrews, 1964).

FIGURE 14

SEPHADEX G-200 CHROMATOGRAPHY OF WILD-TYPE NEUROSPORA FPGS.

Step 3 enzyme (20 mls) of Lindegren A wild-type was applied to a Sephadex G-200 column (2.6 x 80 cm). The column was washed with buffer A. Fractions of 6 ml were collected and assayed for enzyme activity.

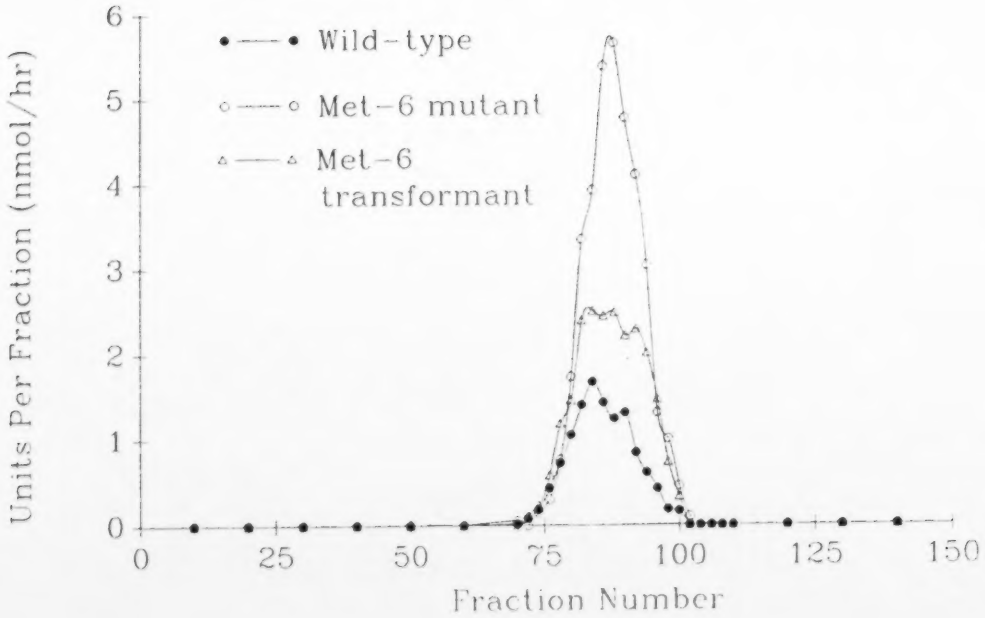
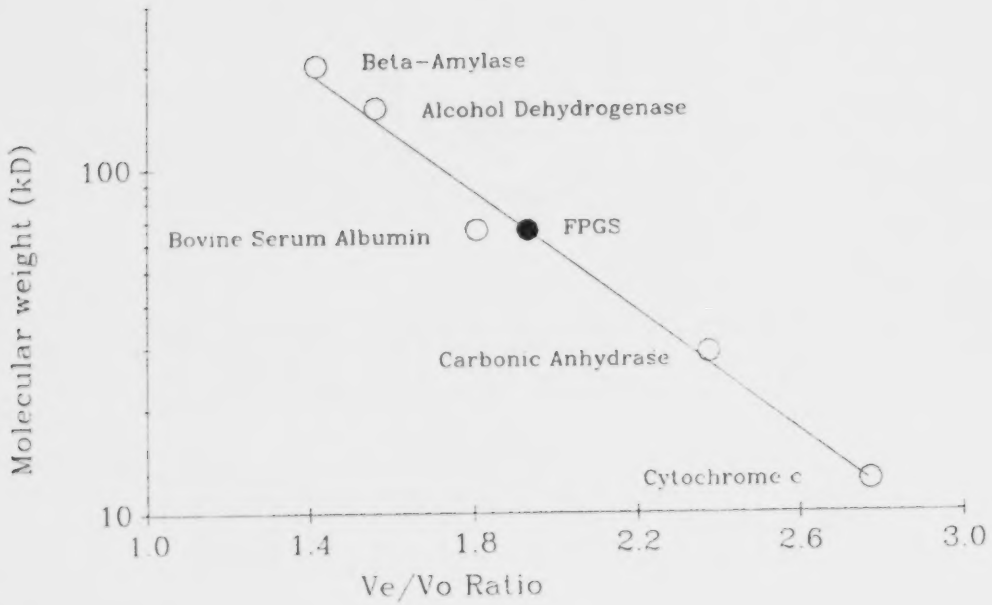

Wild-type
Met-6 mutant
Met-6 transformant
Units Per Fraction (nmol/hr)
Fraction Number
0
1
2
3
4
5
6
0
25
50
75
100
125
150

FIGURE 15

NATIVE MOLECULAR WEIGHT OF WILD-TYPE NEUROSPORA FPGS

A sample of Lindegren A wild type mycelial Step 3 extract was compared against reference proteins on a column of Sephadex G-200 (2.6 x 80 cm) eluted with buffer A. Standard proteins, with their molecular weights, were: Alcohol dehydrogenase (150,000); Bovine serum albumin (66,000); Carbonic anhydrase (29,000); Cytochrome c (12,400). Blue dextran 2000 was used to determine Vo. The equation of the line formed from the standard curve of known molecular weight was $\log(M_r) = -0.8706\ (Ve/Vo) + 3.5002$.


Beta-Amylase
Alcohol Dehydrogenase
Bovine Serum Albumin
FPGS
Carbonic Anhydrase
Cytochrome c
Molecular weight (kD)
100
10
1.0
1.4
1.8
2.2
2.6
3.0
Ve/Vo Ratio

desalted using BioGel P6-DG (Step 4 protein; Table 16).

Further purification of FPGS protein was achieved by applying Step 4 protein to a 1.5 x 10 Reactive Green 5 column pre-equilibrated with buffer A. After loading the protein, the column was washed with 100 ml of buffer A. FPGS activity was eluted by applying a linear KCl gradient (0 to 0.8 M) in buffer A (Table 16).

After concentrating the FPGS fractions by ultrafiltration and desalting through BioGel P6-DG columns, the resulting Step 5 protein was applied to a 2.5 x 10 cm column of Heparin agarose pre-equilibrated with buffer A, pH 7.0. The column was washed with 100 ml of extraction buffer, followed by elution with a 200 ml KCl linear gradient (0 to 0.5 M KCl in buffer A, pH 7.0) (Table 16). The pooled FPGS-active fractions (Step 6 protein) were concentrated and desalted through BioGel P6-DG columns equilibrated and washed with 10 mM potassium phosphate-containing buffer C. The resulting desalted Step 6 protein was applied to a column of hydroxylapatite pre-equilibrated with buffer C. FPGS was not retained under these conditions and was collected in the column wash as Step 7 protein (Table 16). The entire column wash was diluted two-fold with buffer A without glycerol and was applied to a MonoQ-HR 5/5 FPLC column pre-equilibrated with buffer A containing 10% v/v glycerol. A KCl gradient (10 to 600 mM) in this buffer was used, and fractions containing FPGS activity were pooled to give Step 8 protein (Table 16). The protocol for isolation of Neurospora Lindegren A wild-type FPGS (Table 16) resulted in a 56,000-fold increase in specific activity and a final yield of 12%. This is probably an overestimate of yield due to the observation that an FPGS-dependent inhibitor was removed at the ammonium sulfate fractionation step (Cossins and Chan, 1988). Peak enzymatic activity eluted from the FPLC is shown in Figure 16. Sodium dodecyl sulphate gel electrophoresis of the purified enzyme fractions demonstrated 3 silver-stained bands, with sizes of approximately 75 kDa, 60 kDa and 45 kDa (Figure 16). It appears unlikely that the protein of highest or lowest molecular weight observed after SDS-PAGE had FPGS activity as the concentration of either band did not coincide with the peak of FPGS-

Table 16. Partial Purification of N. crassa Lindegren A Wild Type FPGS Protein .

Fractionation Step	Volume (mls)	Activity (unit/ml)	Protein (µg/ml)	Specific Activity (unit/mg)	Purification (x-fold)	Yield
1. Homogenate	1050	5.84	12680	0.4	1.0	100
2. Streptomycin sulphate	1100	5.65	4640	1.2	2.8	101
3. 45-70% Ammonium sulphate	400	93.85	21080	4.4	10.2	673
4. DE-52 cellulose	320	29.95	1720	17.4	40.3	156
5. Reactive Green 5	225	31.84	20	1584.1	3666.6	116
6. Heparin Agarose	180	22.64	4	5670.2	13125.4	66
7. Hydroxylapatite	43	33.60	7	4784.9	11076.2	24
8. FPLC MonoQ-HR5/5	2	242.64	10	24264.3	56166.7	12

FPGS activity is expressed in nmoles of glutamate incorporated into product/hour.

active fractions. Silver staining of the 60 kDa protein band was greatest in fractions with the highest FPGS activity (Figure 16). It therefore appears likely that the peak of FPGS protein occurred at a molecular weight of 60 kDa.

Preliminary characterization of the 56,000-fold purified folylpolyglutamate synthetase was limited due to the instability of Step 8 protein. After MonoQ FPLC chromatography, FPGS activity was unstable and enzyme activity was completely lost after storage at 4°C for 4 days in Buffer A containing 10% glycerol. Step 8 protein had no detectable dihydrofolate synthetase activity and the preparation lacked any ability to glutamyl conjugate dihydropteorate. The preparation was able to incorporate L-glutamate into folylpolyglutamate with both 5,10-methylenetetrahydrofolate mono- and diglutamate. These results indicate that the formation of folylhexaglutamates from the folylmonoglutamate substrate was catalyzed by this purified preparation. It therefore appears likely that both activities, characteristic of the met-6 and mac mutants (equation 4a and 4b of the Introduction), were present. Thus, no definitive evidence has been provided from the purification of wild-type, mutant or transformant extracts to indicate that two independent proteins were responsible for the utilization of the mono- and diglutamate substrates. All attempts to detect a second separate FPGS protein were unsuccessful. The coincidence that both enzyme activities occur in the same peak, combined with a significant lack of distortion of the ratios of the two activities was observed at this late a stage in the purification procedure, suggests that two proteins were not involved. It also appears unlikely that either of the proteins of molecular weight 75 or 45 kDa observed after SDS-PAGE had FPGS activity as both of their concentrations did not coincide with the peak of the FPGS-active fractions.

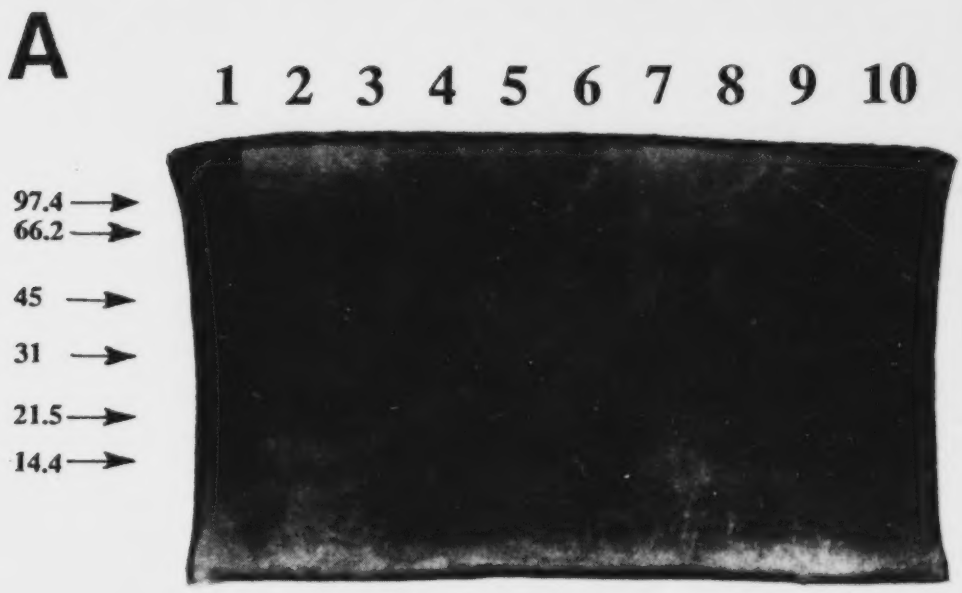
FIGURE 16

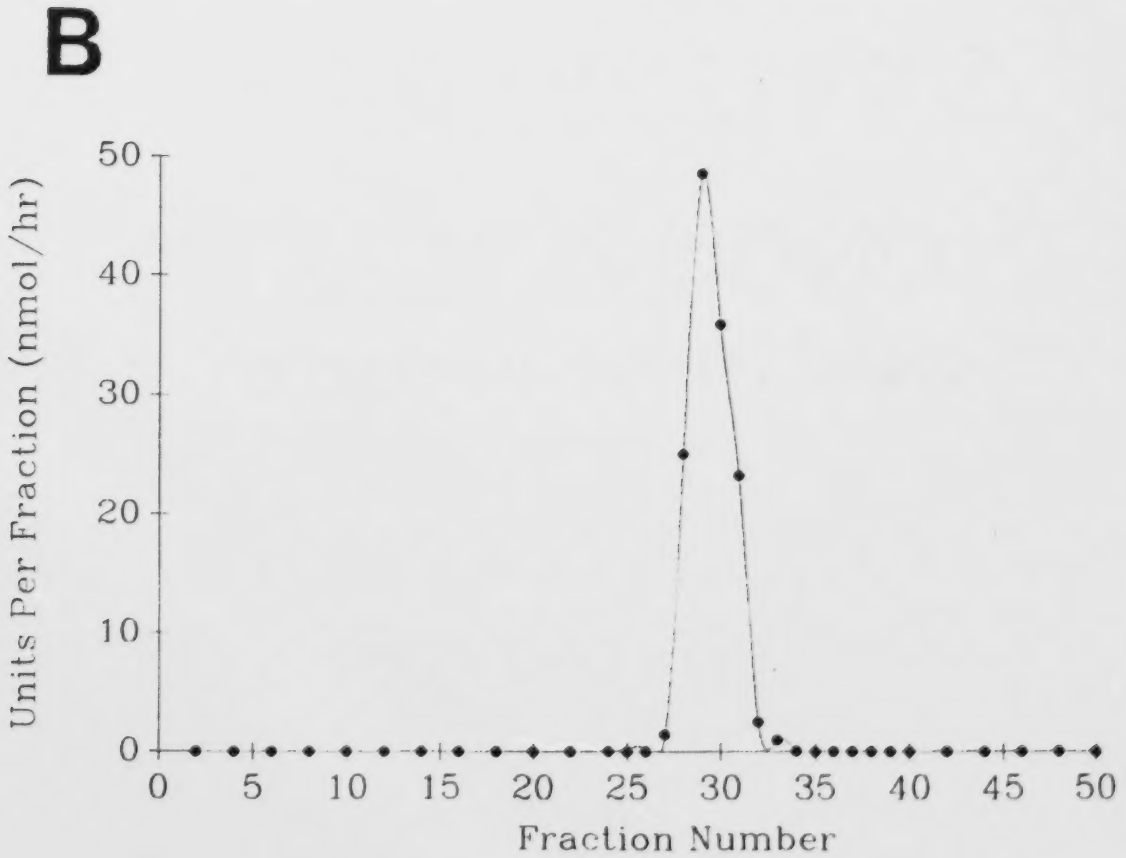
SODIUM DODECYL SULPHATE GEL ELECTROPHORESIS OF PARTIALLY PURIFIED FOLYLPOLYGLUTAMATE SYNTHETASE .

Experimental conditions are described under Materials and Methods.

A. SDS-PAGE. Step 8 Protein was electrophoresed, and detected using BioRad silver staining procedure. Standard molecular weight marker proteins are shown in lane 1. Lane 2, Bovine Serum Albumin as a reference; lane 3-10 Step 8 protein in the highest fractions of FPGS activity, performed in duplicate. Lane 3-4 correspond to FPLC fraction 27 in duplicate. Lane 5-6 correspond to FPLC fraction 28 in duplicate. Lane 7-8 correspond to FPLC fraction 29 in duplicate. Lane 9-10 correspond to FPLC fraction 30 in duplicate.

B. Peak FPGS enzymatic activity of FPLC fractions. Fractions were assayed for enzyme activity as described in the Materials and Methods. FPGS activity is expressed in nmol of L-glutamate incorporated into product per hour. Fractions 27-30 with peak FPGS activity were run on SDS-PAGE as shown above.


A
1 2 3 4 5 6 7 8 9 10
97.4
66.2
45
31
21.5
14.4


B
Units Per Fraction (nmol/hr)
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50
Fraction Number

4. Discussion

The Discussion presented in the following pages has been organized into six major sections. The first deals with analysis of the cloning and transformation of the Neurospora met-6$^+$ gene coding for the folylpolyglutamate synthetase (FPGS) enzyme. Following this, results obtained from nucleotide and amino acid sequences of the met-6 gene are discussed. Next is a characterization of the met-6$^+$ transcript using mRNA and cDNA products to study transcription initiaton, termination, and introns. A discussion of the nature and distribution of in vivo and in vitro polyglutamate derivatives follows. The purification and properties of Neurospora folylpolyglutamate synthetases are then discussed. The Discussion concludes by proposing a name change for what now appear to be three alleles of the met-6 gene, with final comments addressing some questions raised during the course of the research.

4.1 Cloning and Transformation of the Met-6$^+$ Gene

The alleles of the met-6$^+$ gene in Neurospora crassa were examined in the course of this research. The Neurospora mutants strains met-s2706 and mac both complement met-6, but do not complement each other, indicating that at least met-s2706 and mac are alleles. In high resolution recombination studies with flanking markers, met-6 and met-s2706 behaved like alleles, while mac behaved atypically, although exhibiting nearly equal linkage (Murray, 1969). Met-6, met-s2706, and mac mutant loci are known to be closely associated to the met-6$^+$ region, identified to be present between chromosomal markers thi-1 and ad-9 on the right arm of chromosomal linkage group I of N. crassa (Murray, 1969). It was uncertain whether the separate synthetase activities of the mutants (equations 4a and 4b of the Introduction) were associated with one FPGS protein or two. In an initial attempt to investigate FPGS structure and function, research for this thesis examined whether the fully functional FPGS is defined by two different genes or two different functional

domains of the same gene.

Cloning and transformation of the N. crassa met-6$^+$ gene was accomplished by functional complementation of met-6, met-s2706, and mac mutant spheroplasts. Transformation with the met-6 containing cosmid pSV50-4.1.3.1 resulted in prototrophy for all three mutant strains examined. Subcloning revealed that the three methionine auxotrophs associated with FPGS could be complemented to the prototrophic phenotype by a 3.5 kb EcoRI/BamHI fragment of genomic DNA (Figure 3). This fragment was found to contain an ORF encoding a 528 amino acid protein. A 2.2 kb PCR fragment of wild-type 74A genomic DNA, which extended just past the boundaries of this ORF, was also capable of complementation of mutant spheroplasts (Figure 8). Transformation to prototrophy of all three mutants with such small fragments implies that the met-6, mac and met-s2706 mutations are in very close proximity. Thus, the three mutations affect either the same gene or genes that are immediately adjacent to each other. Since regions of homology to foreign FPGS genes were only detected in the long ORF, the second possibility seems unlikely.

Support for actual transformation of the three mutants, rather than simple reversion of mutant loci, was obtained through comparison of previously calculated reversion frequencies with the transformation frequencies generated here. Murray (1969) showed that the met-6 mutant gave one prototrophic revertant and two partial revertants in approximately 6 x 10^7 conidia, whereas mac produced only one revertant in 5.5 x 10^8 conidia. Negative control data (pSV50 transformation of mutant spheroplasts) from this study closely approximated spontaneous reversion rates, while prototrophic transformant colonies using plasmid or cosmid DNA were generated at frequencies of approximately 50 transformants per transformation mixture, or 1 transformant per 2.5 x 10^4 conidial spheroplasts. This demonstrated that the prototrophs arose by transformation rather than reversion. This conclusion was corroborated by Southern analysis, where the presence of additional copies of the met-6$^+$ DNA was confirmed in prototrophic transformants of all three strains.

4.2 Nucleotide Sequence of the Met-6$^+$ Gene.

The 3440 nucleotide EcoRI/BamHI insert of recombinant plasmid pIA50, containing the met-6$^+$ gene, was sequenced and analyzed to determine the number of genes encoded by the insert. It was possible that more than one gene encoding a protein of approximately 66 kDa could be present in the 3.5 kb fragment, either adjacent to each other or in overlapping reading frames. In order to eliminate this possibility, all six possible reading frames of the entire 3.5 kb insert were examined. Computer analysis of this region, excluding intron sequences, resulted in a single open reading frame (ORF) with two potential start sites. Initiation of translation at the first AUG of the ORF would give a protein of 58,672 Daltons; at the second AUG, a protein of 54,646 Daltons. The presence of this ORF in the met-6$^+$ gene fragment of the pIA50 plasmid further supports the notion that the three mutations affect the same gene.

Codon usage in the long ORF resembled that of other Neurospora genes with low levels of expression (Kinnard and Fincham, 1983; Orbach et al., 1986) (Table 9). While the lack of resemblance to highly expressed genes is not sufficient basis to predict levels of gene expression, the codon usage pattern does correlate with the low levels of expression observed with FPGS protein.

Sequence analysis comparing the Lindegren A, 74A, and Emerson wild-type strains revealed sixteen sequence differences between each other in the long ORF (Figure 5; panel 3 through 18; Table 10). Of these differences, all were base substitutions, nine silent at the amino acid level and the remaining seven with significant changes to non-similar amino acids (similar amino acids being: A,G,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W; as defined by PCGENE™ software) (Table 10). These seven significant amino acid alterations did not produce a phenotypic change from the wild-type. The absence of phenotypic change in response to substantive amino acid variations indicates that such polymorphic sites occur in regions of the protein where the precise amino acid sequence

was not essential to protein function, similar to observations seen for the mtr gene in Neurospora (Dillon and Stadler, 1994).

The sequence of the mutant strains showed the most nucleotide homology to Lindegren A wild-type. Each of the mutants have a varied and confusing history of crosses and back crosses to various strains. With the evidence presented, it can be speculated that the mutant versions of the genes were isolated from a Lindegren A-like background. Identification of polymorphisms in wild-type strains other than Lindegren A allowed the elimination of changes at polymorphic sites as potential mutant sites. After such consideration, only one such difference was detected that could be correlated with a mutation. In the met-6 mutant sequence, a T to C transition results in a serine to proline amino acid change. This occurs in a region of the protein which is highly conserved with other species. This change of a single amino acid resulted in the loss of the ability to use 5,10-methylenetetrahydrodiglutamate or longer chain length substrates to form folylhexaglutamates, but did not alter the ability to use 5,10-methylenetetrahydrofolate-monoglutamate to form 5,10-methylenetetrahydrofolatediglutamate. This location was different from the site identified as the SF4 mutation folC gene of E. coli. The SF4 site occurred as a G925A base change resulting in an A309T amino acid change, with a corresponding decrease in long chain polyglutamate production. Using the E. coli FPGS numbering system, the met-6 mutation would occur at amino acid 83 of the E. coli folC protein.

Results from comparison of FPGS sequences have revealed two regions of Neurospora FPGS which were homologous to sequences believed to be nucleotide binding sites (Bognar et al., 1987). The sequence motif of a nucleotide binding site designated an A region (aa 98-107) and the motif of an inverted B region (aa 202-220) (see Section 3.3) are highly conserved across FPGS enzymes (Figure 6, underlined). All FPGS enzymes studied to date have an absolute requirement for divalent cations and ATP. These homologous motifs may represent common structural elements which would be expected to

be associated at or near the active site to facilitate conformation change of the active site upon binding ATP (Bognar et al., 1987).

The database search also revealed that the sequence at the met-6 mutation occurs between the A and B nucleotide binding domains in the primary amino acid sequence; this supports the possibility that the mutation occurs at or near the catalytic centre of the enzyme. Based on this supposition, future work with in vitro mutagenesis of FPGS should focus on identification of the amino acids located at the putative folate binding site.

The exact nature of the mac and met-s2706 mutants was not defined in this study. Near similar ratios of reversion in the met-6 and mac mutants (Murray, 1969) suggest that the mac mutant may also be a point mutation of a single nucleotide. Furthermore, the ability of DNA from one mutant to rescue the others to prototrophy strongly implies that the mutations occur in the same gene.

4.3 The Met-6$^+$ Transcript.

As seen by Northern analysis, the met-6$^+$ transcript is approximately 2.0 kb in length (Figure 7). Although knowledge about the regulation of FPGS expression is limited, the data presented here suggest that the level of the met-6$^+$ transcript varied based on the age of mycelium. Higher transcript levels were detected in freshly germinated tissue, with less expression seen in ungerminated conidia and cultures in stationary phase (Figure 7B). Previous studies have shown that total folate pools and folylpolyglutamate levels increase rapidly as initial seed development proceeds in plants (Roos and Cossins, 1971; Chan et al., 1986). The variation in FPGS enzyme activity during Neurospora culture growth reported by Cossins and Chan (1984) showed that there was a discrepancy between the abundance of the mRNA in this study versus the level of protein activity after 24 hours, suggesting a very short-lived production of FPGS messenger and a stable protein in vivo.

Characterization of the met-6$^+$ transcript using cDNA intermediates was attempted to study the location of introns and transcription initiation-termination sites. A single intron

and a transcriptional termination site were identified; however, definitive identification of the transcriptional start site(s) was not achieved. However, assuming that the 2.0 kb transcript detected by northern analysis is the met-6$^+$ transcript (Figure 7), a 1587 bp ORF with 270 bp of 3' untranslated sequence would give rise to approximately 80 bp of 5' untranslated sequence to account for the full length of transcript.

The translational start site on the transcript also remains to be determined, as two possible AUG start sites were present in the ORF described above. Conceivably, Neurospora FPGS may be analogous to rat fumarase, which has been shown to be synthesized from a single mRNA species by alternative translation initiation at two in-phase AUG codons. This has been postulated to be due to a secondary structure effect which hinders translation efficiency at the first AUG-site of the transcript (Suzuki et al., 1992). A similar situation occurs in the MOD5 gene of yeast, where alternative translation of two in-frame AUG codons is dictated, at least in part, by a translational readthrough of the first AUG of the ORF (Boguta et al., 1994). In both these examples, the differential translation intitation has been shown to be related to subcellular compartmentalization of the translated products. In addition to the differential translation initiation mechanisms described above, subcellular compartmentalization has been shown to occur through cotranslational translocation and mitochondrial processing. A single translation product of the FUM1 fumarase gene of yeast is processed in the mitochondria prior to being distributed between the cytosol and mitochondria (Stein et al., 1994). Evidence from Cossins and Chan (1984) showed that subcellular compartmentalization of FPGS is significant in Neurospora as evidenced by the effect that mutations have upon mitochondrial levels of this enzyme. Neurospora wild-type FPGS activity was principally associated with the cytosolic fraction, with only about 20% of recoverable FPGS activity associated with the mitochondrial fraction (MacDonald et al., 1995). However, the mutants met-6 and mac lack the mitochondrial FPGS activity in addition to missing a cytosolic FPGS activity; the met-s2706 mutant, on the other hand, has the mitochondrial FPGS activity similar in level to the

wild-type but lacks the full cytosolic activity (Cossins and Chan, 1985). Further examination of the two putative AUG translational start sites may elucidate the mechansims of FPGS cytosolic/mitochondrial distribution.

4.4 Labeled and Unlabeled Folylpolyglutamate Pools.

Many previous studies of in vivo folates have been hampered by a lack of a rapid, sensitive, and highly reproducible technique for polyglutamate identification (Mullins and Duch, 1992). Cleavage of folates to the corresponding p-ABAGlu_n derivatives and isolation as Bratton and Marshall reactive derivatives have greatly simplified HPLC separation and characterization of glutamate chain-length through the generation of a homologous series of compounds differing only in glutamate chain length (Shane, 1986). The assignment of glutamate chain length to particular eluted peaks was based upon the near identical elution profiles from similar columns which had been calibrated by synthetic control p-ABAGlu_ns of known glutamate chain length. The HPLC columns in the present study were highly reproducible, with the elution position of a particular derivative varying little as a function of buffer gradient.

Earlier in vivo studies with microbiological assays showed that in Neurospora wild-type mycelia, long chain folylpolyglutamates account for the majority of the folate pool (Selhub, 1970). Later work employing DEAE-cellulose elution showed that the predominant chain length of folate in Neurospora wild-type was the hexaglutamate (Cossins and Chan, 1984). Analyses of labeled and unlabeled Lindegren A wild-type polyglutamate pools (Table 11; Table 12) were in agreement with these earlier assays. Most of the intracellular folates (>95% of the endogenous pool) existed as polyglutamates. The major wild-type folylpolyglutamate found in whole cell extracts was the hexaglutamate derivative, which at 24 hours of growth accounts for over 90% of the polyglutamate pool as determined by analysis of endogenous unlabeled folylpolyglutamates. Hexaglutamates account for just under 75% of the total intracellular folate pool as determined by

incorporation of [^{14}C]p-ABA into folylpolyglutamates. As seen with other species, an individual polyglutamate chain length dominated each folate pool (Brody et al., 1984), with hexaglutamate dominating total cellular folates.

Knowledge of the forms of the endogenous folates may be useful in evaluating the physiological functions of these compounds. Previous studies have shown that native folylpolyglutamates of Neurospora crassa wild-type mycelium are predominantly 5-methyl- and 10-formyltetrahydrofolates (Cossins et al., 1976; Vora and Tamhane, 1966; Swendseid and Nyc, 1958). The present studies show that the majority of folates in whole cell extracts from all subcellular compartments were present as hexaglutamates. Thus, from this data it is not surprising that a majority of the 5-methyl- and 10-formyl- total cellular folates were in the form of 5-methyltetrahydrofolatehexaglutamate and 10-formyltetrahydrofolatehexaglutamate.

Previous studies on the effects of media supplements on polyglutamate labelling (Chan and Cossins, 1980) suggested that exogenous methionine in the media might affect polyglutamate distributions. Therefore, to obtain appropriate control conditions, Lindegren A wild-type and transformant cultures were grown in methionine media. Analyses of polyglutamate distributions of cultures grown with methionine showed very similar distributions to cultures grown without methionine (data not shown). Therefore, when comparisons between mutant and wild-type or transformant cultures were performed, differences in folylpolyglutamate distribution could be attributed to strain polyglutamate differences rather than a methionine effect on polyglutamation.

E. coli synthesizes folylpoly-γ-glutamates of chain length up to Glu_3, and the glutamate chain is further extended by addition of Glu moieties via α-carboxy linkages (Ferone et al., 1986a). The addition of α-linked residues is catalyzed by an enzyme activity that is distinct from FPGS in this organism (Ferone et al., 1986b). Chan et al. (1991) showed the exclusive γ-glutamyl nature of Neurospora polyglutamate products by examination with yeast carboxypeptidase Y. This enzyme cleaves α-glutamyl peptides and

is therefore ineffective against γ-glutamyl folates. This data was reconfirmed with results from the present study, since the HPLC system used here was able to resolve γ-carboxy-linked p-ABAGlu$_n$ from partially α-carboxy-linked derivatives (Ferone et al., 1986a,b). The p-ABAGlu$_n$s (where $n \geq 3$) were present at retention times identical to γ-carboxy linked p-ABAGlu$_n$ standards, further indicating that the polyglutamate pools in Neurospora were all γ-glutamyl folates.

It was not clear why the folate pool of wild-type Neurospora is primarily and almost exclusively hexaglutamate. Folates in mammalian tissues are metabolized by FPGS to polyglutamates of chain lengths considerably greater than those required for folate retention, although the metabolic advantages of this is not clear (Shane, 1989). McGuire et al. (1980) suggested that in rat liver, folylpentaglutamate had strong affinity for FPGS as seen by strong inhibition of polyglutamate formation with the monoglutamate substrate, but the pentaglutamate was a very poor substrate. It was concluded that the free γ-carboxyl group of the pentaglutamate was placed outside the active site, suggesting absolute dimensions to the active site and a predominant length of the polyglutamate chain length. It was also concluded from this work that the enzymes might regulate their own synthesis by end product inhibition. A predominance of the wild-type hexaglutamate and a complete lack of heptaglutamate folates formed under these experimental conditions might be partially explained in this manner, but the metabolic advantages are not yet understood.

In contrast to wild-type data, hexaglutamates in the met-6 and mac mutants represent less than 10% of the total polyglutamate pools, labeled or unlabeled. In these strains, the conjugated folates were mainly di- and triglutamates. Earlier assays (Cossins and Chan, 1984) indicated that the monoglutamyl folates were the major derivatives in the mac mutant. Unfortunately, p-ABAGlu, arising by cleavage of these derivatives, was not sufficiently resolved by the HPLC procedure to allow accurate quantitation of endogenous derivatives by A_{280} measurements. As a result, the A_{280} data provide only the distributions of di- to hexaglutamyl folates. The possible contribution of the monoglutamyl

derivatives to these folate pools was therefore assessed by [^{14}C]p-ABA feeding experiments. These data, in conjunction with that of earlier work (Chan and Cossins, 1980; Cossins and Chan, 1984), confirm that met-6 and mac mutations result in polyglutamate deficiency. The relatively low polyglutamate distribution of met-6 and mac would be an effective limitation on the polyglutamate-dependent transmethylation reaction and cause methionine auxotrophy as a result. The reason for decreased [^{14}C] incorporation in the mutants as compared to the wild-type was not clear. Previous studies on the effect of media supplements on polyglutamate labelling (Chan and Cossins, 1980) suggest that exogenous methionine in the media of the met-6 and mac mutants may account for part of the observed decrease. Furthermore, the absence of long-chained polyglutamates in met-6 and mac might affect cellular retention of labeled folates (McGuire and Bertino, 1981; Kisliuk, 1981) and reduce the amount of [^{14}C] recovered in the harvested mycelia.

Different from met-6 and mac data, but similar to wild-type data, the met-s2706 mutant culture contained polyglutamate derivatives primarily in the form of hexaglutamyl folate. Methionine stimulated the growth of the mutant met-s2706, but the mutant was leaky and permitted growth in minimal medium at a lowered rate of mycelial production than the wild-type. Folate analyses by Cossins and Chan (1985) showed that the mutant generated folylpolyglutamate, but at concentrations that were significantly lower than levels detected in the wild-type. A lack of polyglutamyl folate derivatives formed by the FPGS enzyme might affect normal folate retention by cells (McGuire and Bertino, 1981; Kisliuk, 1981). Therefore, in the case of met-s2706, impaired folylpolyglutamate synthesis could be manifested in a consequent decrease in folate retention. Although the met-s2706 mutant possessed hexaglutamate folates and had a leaky phenotype, it can be postulated that reduced folate levels in the deficient met-s2706 mutant may have caused a decrease in methionine production using Neurospora's folylpolyglutamate-dependent methionine synthase.

Transformation of met-6 and mac spheroplasts resulted in prototrophy and ability to

grow in the presence of benomyl. FPGS pools in cultures of Neurospora mutant transformants were similar to wild-type. These cultures generated long-chained polyglutamates with the major derivative being the hexaglutamate. Over half the endogenous folates in transformants were hexaglutamates, with significant amounts of penta- and tetraglutamates and smaller amounts of lower chain length folates. After a 24 hour incubation with [^{14}C]p-ABA, radioactivity was associated with several folates and subsequent cleavage showed this to be largely hexaglutamate in nature. In these transformant strains however a clear peak of pentaglutamate was also detected. This work suggested that no heptaglutamate was produced under these conditions, in agreement with previous wild-type studies (Cossins and Chan, 1984). The loss of methionine auxotrophy in the Neurospora transformants and the ability to form long-chained polyglutamates in vivo suggests that expression of both FPGS activities (equations 4A and 4B) was present in the transformed tissues.

Transformant studies of both labeled and unlabeled polyglutamate pools presented in this thesis were in agreement with earlier mammalian transfection and transformation studies (Sussman et al., 1986; Garrow et al., 1992; Osborne et al., 1993; Shane et al., 1993; Lowe et al., 1993), where expression of the FPGS gene from a number of sources relieved auxotrophic requirements for end products of one-carbon metabolism in mutant cell lines. The loss of methionine auxotrophy in the Neurospora transformants and the ability to form long-chained polyglutamates in vivo suggests that the FPGS gene was present, confirming transformation. As the result of positive transformations with plasmid pIA50 DNA and the folate synthesized by such transformations, it is therefore concluded that the 3.5 kb EcoRI/BamHI fragment contains the wild-type FPGS gene.

One goal of this study was to determine the percentage distribution of glutamate chain lengths of endogenous and labeled Neurospora tissue folate pools. Total cellular folate recoveries using this technique could have been estimated by the addition of labeled PteGlu standards when endogenous folates were being determined, and by adding

unlabeled PteGlu standards when the distribution of labeled folates was being determined (Shane, 1986). However, the percentage of overall polyglutamate chain length distribution could be assumed to be unaffected based on the premise that losses of folates or p-ABAGlu$_n$s in any one step may be the same irrespective of glutamate chain length .

Although no attempt was made to monitor the kinetics of polyglutamate formation for labeled derivatives, percentage distribution of labeled pools in particular chain lengths were in close agreement with unlabeled folate pools. Such results indicated the polyglutamate forms were near, but not at, steady state distribution by 24 hours of labelling. Under wild-type nutritional conditions, it was clear that exogenous [^{14}C]p-ABA appeared to be approaching equilibration among the various folylpolyglutamates at 24 hours (Table 12), as judged by the closeness of HPLC profiles of radioactively labeled folylpolyglutamate pools to profiles of endogenous folylpolyglutamates Indeed, the steady-state distribution of folate derivatives among the various polyglutamate derivatives is approximately represented by the endogenous profiles and the ^{14}C radioactivity profiles.

4.5 The Purification and Properties of Neurospora FPGS

The physiological importance of the enzyme folylpolyglutamate synthetase is now well established. Previous work has shown that Neurospora appears to contain more than one synthetase activity (Ritari et al., 1973; Cossins and Chan, 1983, 1984, 1985) as seen in the Introduction (equations 4A and 4B). Extract fractionation and heat treatments provided evidence that these cytosolic activities were present in the wild-type. From work on met-6:mac heterokaryons, the individual FPGS activities were found to be complementary and mycelial fusion gave cultures that formed polyglutamates in vivo (Cossins and Chan, 1984; Chan and Cossins, 1984).

Work from this thesis has shown that each of the mutant strains lacked fully functional folylpolyglutamate synthetase activity, resulting in an impairment of folylpolyglutamate synthesis. In terms of overall deficiency in polyglutamate synthesis,

each mutant strain possessed only partial FPGS activity. FPGS assays of Step 3 protein indicated that met-6 lacked the cytosolic activity present in the wild-type, mac and met-s2706 mutants that utilized $H_4PteGlu_2$, or more effectively 5,10-CH_2-$H_4PteGlu_2$, to form higher polyglutamates (equation 4B). FPGS assays also indicated that both met-s2706 and mac mutants lacked the activity present in the wild-type and met-6 mutant which utilized $H_4PteGlu$ or 5,10-CH_2-$H_4PteGlu$ (equation 4A), showing that cytosolic met-s2706 enzyme resembled the cytosolic mac enzyme. A further similarity between mac and met-s2706 was the presence of activity that added glutamate to the diglutamate substrate.

Polyglutamate synthesis to the level of hexaglutamates seen in met-s2706 tissue extracts could not be accounted for by a cytosolic activity unable to utilize the monoglutamate substrate, as seen by these FPGS assays. Since there was no significant difference between the cytosolic FPGS activity of the mac and met-s2706 mutants, any difference accounting for the presence of hexaglutamates in tissue extracts may be associated with a mitochondrial FPGS activity (Cossins and Chan, 1985), present in the met-s2706 mutant, but absent in the mac mutant. This defines a two-step process which would necessitate exchange of folate between cytosol and mitochondria. The synthesis of hexaglutamates in this manner would account for the presence of limited hexaglutamates in met-s2706 tissue extracts, and the leaky nature of the met-s2706 mutant that can grow slowly in the absence of methionine.

In common with the wild-type, Step 3 protein from the transformants was able to utilize both of the folate substrates. On the other hand, met-6 and mac/met-s2706 used only 5,10-CH_2-$H_4PteGlu_1$ or 5,10-CH_2-$H_4PteGlu_n$ respectively. Although the chain lengths of the polyglutamates formed from these reaction systems were not analyzed it follows from earlier work (Cossins and Chan, 1984; Chan et al., 1991) that the wild-type FPGS protein would form 5,10-CH2-$H_4PteGlu_n$ (n=3-5) from the diglutamate substrate. It may be assumed that the transformants converted 5,10-CH2-$H_4PteGlu_2$ into polyglutamates with at least three glutamate residues. It is, therefore, concluded that the

DNA insert of plasmid pIA50 used in these transformations allowed the expression of FPGS protein whose activity was complementary to that expressed by either mutant.

When samples of Step 3 protein were chromatographed on Sephadex G-200, it was clear that wild-type and transformant FPGS protein had an apparent molecular weight of 66,000 ± 4,000 Daltons. These gel filtration studies provided evidence for a single peak of FPGS activity catalyzing the production of labeled polyglutamate synthesis from 5,10-CH_2-H_4PteGlu_1 and its diglutamate. Thus, no direct support was evident in these strains for the synthesis of polyglutamate catalyzed by separate proteins of distinct molecular size. The apparent molecular weight values for FPGS protein isolated from the mutants also averaged 66 kDa. This implied that each mutation, although affecting the nature of the FPGS reaction, had little apparent effect on the overall size of the native protein. In addition, the similarity of mutant and wild-type activities during gel filtration studies in the presence of ß-mercaptoethanol suggest that the structure of the proteins was very similar. In spite of the fact that partially purified mutant enzymes lack full synthetase activity, the size and/or shape of each of the mutant FPGS proteins appears to be similar.

The protocol used to purify folylpolyglutamate synthetase from Neurospora Lindegren A wild-type was modelled after the protocol developed by Chan et al. (1991), which gave a partial purification of 4100-fold. This method, as well as similar purification methods for bacterial and mammalian synthetases (Masurekar and Brown, 1975; Shane, 1980; Bognar and Shane, 1983; Pristupa et al., 1992; Moran and Colman, 1984) involved ammonium sulfate fractionation followed by one or more gel filtration, ion exchange, and affinity chromatography steps. All of these protocols attempted to overcome enzyme instability by the addition of one or more agents to the extracts, including ß-mercaptoethanol, ATP, glycerol, and protease inhibitors. The new procedure used here allowed purification of the enzyme to near homogeneity. As a result, folylpolyglutamate synthetase activity was purified more than 56,000-fold with a yield of 12%. As mentioned in the Results section, this is probably an overestimation of yield due to the observation that

an FPGS-dependent inhibitor was removed at the ammonium sulfate fractionation step (Cossins and Chan, 1988). The preparation obtained after Mono-Q chromatography contained three bands, with a primary band of an approximate molecular weight of just under 60 kDa after sodium dodecyl sulfate polyacrylamide gel electrophoresis. This value was in close agreement with earlier gel filtration analyses of this protein from the wild-type, discussed above. Preliminary characterization of the 56,000-fold purified folylpolyglutamate synthetase was limited due to the instability of the protein.

FPGS protein as recovered by this protocol lacked ability to glutamyl conjugate dihydropteorate, suggesting that the dihydrofolate synthetase and folylpolyglutamate synthetase enzymes were not complexed as in E. coli (Bognar et al., 1985). This was in agreement with conclusions by McDonald et al. (1995) that these were two separate proteins encoded by separate genes. The reason for the fundamental differences in the structural organization of bacterial and fungal DHFS/FPGS activities is unclear, and consequently this basic area of folate biochemistry warrents further investigation.

The highly purified preparation from the wild-type was able to incorporate L-glutamate into folylpolyglutamate using both 5,10-methylenetetrahydrofolate mono- and diglutamate substrates. These results indicate that this purified protein was still able to catalyze the formation of folylhexaglutamates from the folylmonoglutamate substrate. Therefore, both of the activities associated with the mutants (equation 4A and 4B of the Introduction) were present in the same preparation. The ratios of the two activities also remained relatively constant throughout the purification procedure, suggesting that only one protein was involved. Thus, we could find no direct support for the possibility that polyglutamate synthesis in these strains was catalyzed by separate proteins.

Purification studies suggest that the met-6, met-s2706 and mac mutations probably occur within a single, FPGS-encoding gene. Selhub (1970) suggested that two proteins gave rise to the two activities described; however no definitive evidence has been obtained to indicate that two genes produce two independent proteins which are responsible for the

utilization of the mono- and diglutamate substrates in equations 4A and 4B of the Introducion. All attempts at detecting a second separate cytosolic FPGS protein have been unsuccessful. The coincidence that both enzyme activities occurred in a single peak of molecular weight fractions with a lack of distortion of the ratios of the two activities during significant purification suggested that only one enzyme was involved.

4.6 Suggested Name Change for the Mutant Loci of the Met-6 Gene

As concluded from Sections 4.1 to 4.5 above, the results of both biochemical and genetic studies have lead to the conclusion of this thesis that the met-6, mac and met-s2706 mutations affect a single gene for folylpolyglutamate synthetase. The mutant deficiencies would therefore be seen as changes to a single enzyme, not different enzymes. As a result, the met-6, mac and met-s2706 mutants would be more appropriately renamed as alleles of a single gene. It is proposed that the mutant designated met-6 be renamed met-6-1, since it was the first designated mutant loci at the met-6 locus. The mutant designated met-s2706 should be renamed met-6-2 and the mutant designated mac should be renamed met-6-3 upon confirmation of this single enzyne hypothesis.

4.7 Suggestions for Future Study

Throughout the course of this project, many avenues for future research into folate metabolism have been revealed. The points below represent a sampling of interesting questions which have yet to be examined.

The characterization of bacterial and mammalian folylpolyglutamate synthetases has been complicated by the low abundance of this protein and the low yields obtained after purification (McGuire and Coward, 1984). Folylpolyglutamate synthetase represents about 0.01% of the soluble protein in extracts of E. coli , yet levels of up to 4% soluble protein have been detected with plasmid transformants (Bognar et al., 1985). This amplification has allowed purification to homogeneity and characterization of the protein.

Characterization of the wild-type Neurospora enzyme in this study has been hampered by the absence of sufficient protein for complete purification and characterization. Overexpression of the met-6^{+} FPGS protein in a system like E. coli would conceivably allow more detailed studies. Such characterization could extend the above observations and provide support for the suggestion that FPGS protein is encoded by a single gene.

Recently, data from E. coli and Corynebacterium sp. showed that DHFS and FPGS are associated as bifunctional proteins (Bognar et al., 1985; Shane, 1980). On the other hand, separate FPGS and DHFS genes have been isolated from yeast, and the genes have been localized to separate chromosomes (Shane, personal communication cited in MacDonald et al., 1995). The separation of DHFS and FPGS activity in Neurospora mycelial extracts (McDonald et al., 1995) suggested that these enzymes were not complexed as in E. coli. The reasons for the fundamental differences in structural organization are unclear. The production of distinct proteins may facilitate independent control of DHFS and FPGS protein synthesis, regulating folate and folylpolyglutamate levels. This postulation is to date unconfirmed.

The mitochondrial FPGS of Neurospora is also of interest. The procedure used to isolate the cytosolic FPGS protein in this thesis effectively removed a mitochondrial FPGS protein, which precipitated with ammonium sulphate at 0-35% of saturation (Cossins and Chan, 1983). However, the isolation of a significantly purified mitochondrial enzyme product from Neurospora has not yet been attempted. Since mutants of Neurospora FPGS activity affect both cytosolic and mitochondrial FPGS activity, localization of FPGS in Neurospora mitochondria may provide a role in the generation/control of the mitochondrial folate pool, as was seen with folate pools of the met-s2706 mutant. In the Chinese hamster ovary mutant AUXB1, cells lack significant cytosolic and mitochondrial FPGS activity, and contain only folylmonoglutamates. AUXB1 transfectants (AUXB1-coli) expressing the E. coli FPGS gene metabolize cytosolic folates to triglutamates but lack mitochondrial folylpolyglutamates and remain auxotrophic for glycine (Lowe et al., 1994). Addition of a

mitochondrial leader sequence targeting E. coli FPGS to the mitochondria of these cells restored mitochondrial polyglutamate pools and overcame the glycine requirement (Lin and Shane, 1994). Based on this data, the interrelationship of cytosolic and mitochondrial FPGS enzymes appear to be closely associated. Regulation of compartmented folylpolyglutamate pools by competition between mitochondrial and cytosolic FPGS activities might have a role in overall one-carbon metabolism, but this remains yet undiscovered.

Other levels of control that need to be studied are enzyme turnover as well as translational control. These studies could lead to a better understanding of how FPGS is regulated in both the de novo and salvage pathway for folate synthesis. The role that folylpolyglutamate synthetase and γ-glutamyl hydrolases play in the turnover of the polyglutmate pool has yet to been determined. Which signals increase or decrease synthesis of these enzymes, and whether these signals affect polyglutamate chain length in the different compartments of the cell are questions which warrent further work. It has been proposed that changing physiological or nutritional conditions of the cell can alter the glutamate chain length of the folates (Krumdieck et al., 1977; Chan and Cossins, 1980). Signals that affect each of these enzymes might regulate one-carbon metabolism in ways yet unexplained.

In a related area, the FPGS inhibitor (Cossins and Chan, 1988) was seen to be associated with the ammonium sulfate precipitation at the 30-45% fraction. The inhibitory action of this protein may be a direct consequence of interaction with FPGS, and this may have great significance in terms of folylpolyglutamate control. Further regulatory control of folylpolyglutamate homeostasis might be elucidated from further studies.

The present studies suggest that the met-6, met-s2706 and mac mutations occur in a single, FPGS-encoded gene. The change in the met-6 nucleotide sequence occurs as a single point mutation, changing a single amino acid. In addition, due to strong blocks of homology between organisms, the site of the met-6 mutation appears to be conserved

throughout all FPGS genes studied to date and shows limited similarity to a folate binding site. Site-directed mutagenesis of the E. coli folC gene has shown that single amino acid substitutions can profoundly affect the affinity of bacterial FPGS for glutamate and various folate substrates (Keshavjee et al., 1991; Pyne and Bognar, 1992). Similar studies of the Neurospora met-6^{+} gene now appear warranted.

The nature of the dihydrofolate synthetase gene sequence merits comment. Recently, Shane's group sequenced the dihydrofolate synthetase gene in Saccharomyces cerevisiae (personal communication). The predicted amino acid sequence of the gene product showed 20.5% identity with the predicted protein product of the largest open reading frame identified as the FPGS gene in the present work. The dihydrofolate synthetase gene performs a similar reaction to FPGS, the addition of an L-glutamate to dihydropteroate as seen in Figure 2 and Table 2 of the Introduction. Homologous sequence between Neurospora FPGS and yeast DHFS was observed in the region of the A and B nucleotide binding sites. Homologous sequence between DHFS and FPGS was also seen at the site of the met-6 mutation, which was $GX_2TTPHLX_4DS$ in the yeast DHFS. The similarity between FPGS and DHFS gene sequence at this locality may be associated with similar protein function. If the speculation that the FPGS sequence at the site of the met-6 mutation was at or near to the active site, this would imply that the homologous sequence of the DHFS sequence might also be associated at or near the DHFS active site. Future work with in vitro mutated versions of DHFS should enable a more accurate identification of the postulated dihydropteroate binding domain for DHFS, and may give further information and added insights on FPGS as well. It may also be possible that other folate enzymes in the one-carbon pathway may show sequence homology at similar sites and such enzymes therefore provide further avenues for investigation.

Over the last few years, a considerable amount of information has been obtained on the mechanism by which mammalian and bacterial folylpolyglutamates are synthesized, on

the manner in which this synthesis is regulated, and on the effects of the polyglutamate chain on the interaction of folate substrates and inhibitors with folate-dependent enzymes. There is however relatively little information on these topics in fungal systems. Despite this, filamentous fungi like Neurospora are amenable to study by methods of modern molecular biology. It appears from work described in this thesis that further, more elaborate studies of folate biochemistry with this species are feasible and worth undertaking.

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